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**DIFFERENTIAL MECHANISMS OF NUCLEAR RECEPTOR REGULATION
BY THE COACTIVATOR RAC3**

A DISSERTATION PRESENTED

BY

CHRISTOPHER LEO

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

In Partial Fulfillment of the Requirements for the Degree of:

DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY AND MOLECULAR TOXICOLOGY

October 12, 2000

Dissertation submitted October 23, 2000

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DIFFERENTIAL MECHANISMS OF NUCLEAR RECEPTOR REGULATION BY THE COACTIVATOR RAC3

October 12, 2000

Christopher Leo

B.A., College of the Holy Cross, 1993

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Directed by J. Don Chen, Ph.D.

ABSTRACT

The steroid/thyroid hormone receptor superfamily is a large class of ligand-dependent transcription factors that plays a critical role in regulating the expression of genes involved in a broad range of physiological functions, including development, homeostasis, and reproduction. In the absence of cognate hormone, several receptors are able to repress transcription below the basal level via the recruitment of the nuclear receptor corepressors SMRT and NCoR. Upon hormone binding by the receptor, the corepressor complex is dissociated and a coactivator complex is subsequently recruited. This thesis details the mechanisms by which receptor-associated coactivator 3 (RAC3) interacts with nuclear receptors, particularly the vitamin D, estrogen, and retinoid

receptors, and modulates their transcriptional activity. It was discovered that these receptors interact with different α -helical LXXLL motifs of RAC3 in vitro. Mutation of specific motifs differentially impairs the ability of RAC3 to enhance transcription by the receptors in vivo. In addition, the intrinsic transcriptional activation function of RAC3 was also characterized. Here, a single LXXLL motif, NR box v, was found to be essential to activation by serving as a binding surface for the general transcriptional integrator CBP/p300. Finally, the cofactor binding pocket of retinoid receptors was characterized. It was demonstrated that, to a large extent, the coactivator pocket of RAR α overlaps with the corepressor pocket, with the exception of helix 12, which is required for coactivator, but not corepressor binding. Recruitment of RAC3 or SMRT also correlates directly with the ability of RAR α to activate or repress transcription, respectively. Intriguingly, it was discovered that the AF-2 domain of RXR α inhibited cofactor binding to RXR α heterodimers, for deletion of this domain dramatically enhanced RAC3 and SMRT binding. In addition, it was demonstrated that the RXR α cofactor binding pocket contributed minimally to recruitment of cofactors. Conversely, the AF-2 domain of the partnering monomer and its cofactor pocket were required for these interactions. These findings suggest that the partner of RXR α is the primary docking point for cofactors at RXR α heterodimeric complexes. Taken together, this work contributes significantly to the field of nuclear receptor function in detailing the mechanisms by which the coactivator RAC3 is recruited to nuclear receptors and regulates their transcriptional activity.

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ABBREVIATIONS

ACTR	activator of thyroid receptor
AD	activation domain
AF-1/AF-2	activation function 1/2
AIB1	amplified in breast cancer
AR	androgen receptor
bHLH	basic helix-loop-helix
CARM1	coactivator-associated arginine methyltransferase 1
CBP	cAMP response element binding protein
DBD	DNA binding domain
DR	direct repeat
DRIP	VDR-interacting proteins
ER β	estrogen receptor beta
ERE	estrogen response element
GR	glucocorticoid receptor
GRIP1	glucocorticoid receptor-interacting protein 1
GST	glutathione-S-transferase
HAT	histone acetyltransferase
HDAC	histone deacetylase

LBD	ligand binding domain
MR	mineralcorticoid receptor
NCoA	nuclear receptor coactivator
NCoR	nuclear receptor corepressor
NR box	nuclear receptor binding box
PAS	Per-ARNT-Sim domain
PCAF	p300/CBP associated factor
pCIP	p300/CBP cointegrator-associated protein
PGC-1	peroxisome proliferator activated receptor coactivator 1
PPAR	peroxisome proliferator activated receptor
PR	progesterone receptor
RAC3	receptor-associated coactivator 3
RAR α	retinoic acid receptor alpha
RID	receptor-interacting domain
RXR α	retinoid X receptor alpha
SMRT	silencing mediator for retinoic acid and thyroid hormone receptors
SRA	steroid receptor RNA activator
SRC	steroid receptor coactivator
TIF2	transcription intermediary factor 2
TR	thyroid hormone receptor

TRAP	TR-associated proteins
VDR	vitamin D receptor
VDRE	vitamin D response element

CHAPTER I

BACKGROUND

A. Steroid/Nuclear Receptors and Hormone Action

Steroid hormones are small lipophilic molecules that elicit a wide range of biological responses through interaction with the steroid/nuclear hormone receptor superfamily, whose members act as ligand-dependent transcription factors that regulate the expression of hormone target genes. The process by which these molecules carry out their physiological activity is a multi-step one that begins with the arrival of the steroid at its target cell via a plasma transport protein. Upon dissociation from the plasma protein, the hormone is able to diffuse through the cell membrane due to its lipophilic character, where it will bind to the receptor in the cytoplasm or continue through the nuclear membrane to interact with a receptor in the nucleus (1). Cytoplasmic receptors are then able to translocate to the nucleus, where they bind specifically to cognate hormone response elements (HREs) in the promoters of target genes. It is believed that those receptors that are located primarily in the nucleus are constitutively bound to their response elements, for they are able to repress transcription below the basal level in the absence of ligand. Hormone binding by all of these receptors converts them into transcriptional activators that enhance the expression of target genes by mechanisms that include the recruitment of coactivator molecules, which will be discussed in detail in this work.

The steroid/nuclear receptor superfamily can be further subdivided into three classes of nuclear receptors. Type I or steroid receptors include those for estrogens (ER), progestins (PR), androgens (AR), glucocorticoids (GR), and mineralcorticoids (MR). Type I receptors are coupled to heat shock proteins and sequestered to the cytoplasm in the absence of ligand (1). Upon hormone binding, they dissociate from the heat shock proteins, homodimerize, and translocate to the nucleus, where they bind response elements consisting of palindromic repeats (2). The ER recognizes a half-site with the sequence AGGTCA while the GR, MR, PR, and AR all recognize the same element, which contains the sequence AGAACA as the half-site. This observation raises the question of specificity, which may be explained by differential expression of the receptors themselves. It is also possible that residues flanking the half-site contribute to the recruitment of specific receptors to target gene promoters. Type II receptors include those for all-trans retinoic acid (RAR), thyroid hormone (TR), and vitamin D (VDR). These receptors are strictly nuclear and form heterodimers with the retinoid X receptor for 9-cis retinoic acid (RXR). They also bind constitutively to response elements consisting of direct repeats. Intriguingly, all of these Type II receptors recognize the same half-site, which contains the sequence AGGTCA (3). Specificity is determined by the 1-to-5 rule, in which the spacing between each half-site determines which heterodimer will bind (4). For example, the HREs for the VDR, TR, and RAR are composed of direct repeats spaced by 3, 4, or 5 nucleotides respectively. A third class of nuclear receptors is the orphan receptors, so-called because endogenous ligands for these proteins are currently unknown.

The target genes that are regulated by these receptors are involved in a wide array of biological activities including cell proliferation, differentiation, development, reproduction, and homeostasis (5, 6). For example, studies on vitamin A-deficient (VAD) animals have demonstrated that retinoids are required during pre- and postnatal development and in adult life (7). They are indispensable for growth, reproduction, and for the maintenance of numerous tissues. These studies were confirmed upon examination of RAR null mutant mice. Mice lacking RAR α or RAR γ displayed similar defects as VAD animals, including poor viability, growth deficiency, and male sterility (8-10). RAR double knockout mice had a much more severe phenotype, as evidenced by a dramatic reduction in viability (9). These studies demonstrated that retinoic acid is the active vitamin A derivative and that the effects of vitamin A in vivo are mediated by RARs.

The physiological functions of vitamin D and the VDR are also fairly extensive (11). The traditional action of vitamin D, as carried out via its metabolite 1,25-dihydroxy-vitamin D, is to effect calcium and phosphate homeostasis to ensure the deposition of bone mineral. Vitamin D stimulates calcium and phosphate absorption in the intestine and reabsorption in the kidney and bone as to increase the concentration of these ions in the blood. These responses are elicited through target genes that include osteocalcin, osteopontin, and vitamin D hydroxylase (11). Insensitivity to vitamin D, known as hereditary hypocalcemic vitamin D-resistant rickets (HVDRR), results in a failure to achieve normal bone density and has been linked to mutation of the VDR in virtually all cases (12). This strongly suggests that the biological effects of vitamin D are

mediated by the VDR, as with retinoids and RARs. Furthermore, VDR knockout mice displayed a phenotype very similar to HVDRR (13, 14). At various intervals after birth, the mice acquired low bone mass, hypocalcemia, and elevated 1,25-dihydroxy-vitamin D levels. These effects could be partially rescued with a diet of high levels of calcium and phosphate. Consistent with the broad biological roles for nuclear receptors, other targets for vitamin D action have been revealed. For example, vitamin D is thought to be critical to the differentiation of hematopoietic cells such as HL-60 cells, which are a human leukemia cell line that differentiates into macrophage-like cells upon hormone treatment (15, 16). Similarly, vitamin D appears to affect the maturation and proliferation of various normal and neoplastic epithelial cells found in several tissues including the mammary gland, colon, and prostate (11).

The primary actions of estrogens occur in the female reproductive tract. These responses are elicited through target genes that include the progesterone receptor, prolactin, and c-fos, each of which contains an estrogen response element in their promoters and whose expression levels are increased by estrogen (6). In ER α knockout mice (ERKO), female reproductive organs such as the uterus, ovary, and mammary gland developed fairly normally during the pre- and neonatal stages (17). However, development was severely impaired after puberty, indicating insensitivity to estrogen in these tissues. Somewhat surprisingly, male mice showed abnormalities in spermatogenesis and sexual behavior, suggesting an important role for ER α even in male animals (18). More recently, ER β null animals (BERKO) were generated (19). Female mice were fertile, but showed reduced ovarian function and decreased litter size. Male

mice were normal until old age, when hyperplasia of the prostate was noted. These observations are supported by the increased ER β versus ER α levels in prostate and ovary while increased ER α levels were found in the uterus, vagina, and mammary gland (20). Furthermore, the α and β knockout mice display opposite responses to estrogen in the uterus. The ERKO mouse lost responsiveness while the BERKO mouse demonstrated increased response to estrogen as evidenced by increased cell proliferation (21). Therefore, ER β may play an antiproliferative role in the uterus and possible function to counteract the activity of ER α .

Most members of the nuclear receptor superfamily share a common domain structure (Fig. 1). The N-terminus contains the variable A/B region, which also includes the ligand-independent activation function-1 (AF-1) domain. The C region represents the highly conserved DNA-binding domain (DBD) and is followed by the hinge region (D) and the C-terminal ligand-binding domain (LBD) (E). The LBD consists of 12 α -helices, with helix 12 representing the ligand-dependent activation function-2 (AF-2) domain. The LBD also mediates dimerization of nuclear receptors. In the absence of ligand, several nuclear receptors are able to repress basal transcription via recruitment of the nuclear receptor corepressors SMRT and NCoR (Fig. 2) (22, 23). SMRT and NCoR are found in complexes with the corepressor mSin3 and histone deacetylases (HDACs), suggesting that transcriptional repression by nuclear receptors may involve histone deacetylation (24-26). Ligand binding triggers the release of these corepressors and subsequent recruitment of coactivators through a drastic conformational change in the AF-2 domain of the receptor. Structural studies have demonstrated that helix 12 within

the AF-2 domain projects away from the LBD in the unliganded structure (27-30). This helix rotates nearly 180° to pack tightly against the LBD upon hormone binding in the RAR, TR, and ER crystal structures. This conformational change, together with ligand-induced changes in helices 3-5, is believed to facilitate interactions of the receptor with coactivators (31-34). Subsequently, coactivators are able to enhance transcriptional activation by the receptor via mechanisms that include recruitment of the general coactivator CBP/p300 and histone acetylation.

B. Steroid/Nuclear Receptor Coactivators

One of earliest indications that suggested the existence of coactivators for steroid/nuclear receptors was the phenomenon of squelching, in which the presence of a given, activated nuclear receptor interfered with or inhibited transcriptional activation by a second, activated receptor in transient transfection assays. These observations led to the hypothesis that a limiting pool of cellular factors regulated the overall activity of receptors (35). Subsequent biochemical and expression cloning approaches identified a large number of factors that were capable of interacting with steroid/nuclear receptors in a ligand-dependent manner. Many of these factors were also demonstrated to enhance the transcriptional activation function of the receptors as well, and thus were termed coactivators. Prominent among these coactivators was a class of homologous proteins of 160 kilodaltons, the steroid receptor coactivator (SRC) family (36).

The first member of the SRC family, steroid receptor coactivator-1 (SRC-1), was cloned by using the PR-LBD as bait in a yeast-two-hybrid screen of a human B-cell cDNA library (37). SRC-1 interacts in a ligand-dependent manner with and enhances AF-2 transcriptional activation by a broad range of nuclear receptors, including PR, ER, TR, RXR, GR, and PPAR. Recent data also detail the enhancement of ER (38) and AR (39-41) AF-1 activities by SRC-1. In addition, SRC-1 has been demonstrated to interact with the general transcription factors TBP and TFIIB, although the functional consequences of these interactions are unknown (42, 43). Furthermore, SRC-1 is able to enhance transcriptional activation mediated by NF- κ B, SMAD3, and AP-1 (44-46), supporting a role for nuclear receptor coactivators in multiple intracellular signaling pathways. Subsequent studies have identified two functionally distinct SRC-1 isoforms, SRC-1a and SRC-1e, which contain unique C-termini, suggesting that alternative splicing may regulate SRC-1 function(47).

The identification of transcription intermediary factor 2 (TIF2) and GR-interacting protein 1 (GRIP1) established the SRC family of coactivators (48, 49). TIF2 was isolated in a Far-western screen as an ER- and RAR-interacting factor while GRIP1 was isolated using the GR-LBD as bait in a yeast-two-hybrid screen. TIF2 and GRIP1 share 94% amino acid identity, thus represent the human and murine orthologs, respectively. TIF2 and GRIP1 associate in vivo with hormone-bound RAR, ER, and PR and coactivate ligand-dependent transactivation. Like SRC-1, GRIP1 has been demonstrated to enhance receptor AF-1 activity in addition to that of the AF-2 domain (41). A biological role for GRIP1 has been outlined in terms of muscle cell-specific gene expression and

differentiation (50). GRIP1 is overexpressed during muscle cell differentiation and is required for induction and activation of the transcription factors myogenin and MEF-2. GRIP1 interacts with these two factors directly and enhances their transcriptional activation function. Intriguingly, the inv(8)(p11q13) chromosomal translocation results in a fusion between TIF2 and the MOZ gene, which contributes to the pathogenesis of acute myeloid leukemia (AML), suggesting a role for transcriptional regulation by nuclear receptor coactivators in these leukemias (51).

The third member of the SRC family was reported simultaneously by several groups as an RAR-interacting protein (RAC3), a CBP-interacting protein (p/CIP), a hRAR β -stimulatory protein (ACTR), a gene amplified in breast cancer (AIB-1), and a TR-interacting protein (TRAM-1) (52-56). p/CIP represents the mouse homolog while RAC3/ACTR/AIB-1/TRAM are human isoforms. In addition to coactivating many nuclear receptors, pCIP has also been demonstrated to enhance the activity of interferon- α and cAMP regulatory element binding protein (CREB), suggesting that this coactivator may be involved in multiple signaling pathways (53). Furthermore, RAC3/TRAM-1 expression can be upregulated by hormone treatment, which represents another possible mechanism by which coactivators may potentiate hormone action (57, 58). The 26S proteasome also has been demonstrated to regulate RAC3 expression, in addition to the levels of SRC-1, TIF2, and CBP (59). The proteasome inhibitor MG132 enhanced coactivator levels in HeLa cells, suggesting that coactivators may be the target of ubiquitin-mediated degradation and that protein turnover may contribute to their transcriptional activity.

Although many other steroid/nuclear receptor coactivators have been cloned (see Discussion), the SRC family has been the focus of the most intense analysis in recent years. Much evidence exists that supports the role of these cofactors in mediating the transcriptional activation function of the receptors. Furthermore, a biological role for SRC members in hormone action has been revealed with the generation of SRC-1 and RAC3 null mutant mice (60, 61). Since RAC3/AIB-1 is also amplified in breast and pancreatic cancers, these coactivators are likely critical to cell growth and differentiation. Therefore, understanding the precise mechanisms by which these cofactors interact with receptors and modulate their function will contribute greatly to our knowledge of hormone-related human diseases and also serve as a potential target for novel therapeutics.

C. Mechanisms of Nuclear Receptor Interactions with Cofactors

The SRC family of coactivators also shares a common domain structure, with the most highly conserved region being the N-terminal bHLH-PAS domain (Fig. 3A). The bHLH region functions as a DNA-binding or dimerization surface in many transcription factors, including the MyoD family of proteins (62, 63). The PAS motif is also found in several transcriptional regulators, including Period (Per), Aryl hydrocarbon receptor (AhR), and single-minded (Sim). Similar to the bHLH domain, the PAS domain also plays a role in protein-protein interactions and dimerization. However, the function of the bHLH-PAS domains of SRC coactivators remains unknown, though it is likely to

mediate intra- or intermolecular interactions. This bHLH-PAS domain is followed by a centrally located receptor-interacting domain (RID) and C-terminal transcriptional activation domain (AD).

The RID of SRC coactivators mediates ligand-dependent, direct interactions with nuclear receptors (57, 64, 65). Detailed analysis of the sequence of the RID identified a conserved motif, LXXLL, where L is leucine and X is any amino acid, that is termed the NR box (66). Three such motifs are found in the RID of SRC coactivators with an additional, non-conserved NR box also present at the C-terminus of the SRC-1 isoform SRC-1a (Fig. 3B). Site-directed mutagenesis and peptide competition experiments have provided strong evidence for the requirement of these motifs for mediating interactions between coactivators and liganded nuclear receptors (53, 66-68). Further support for the role of these motifs in mediating agonist-dependent interactions with nuclear receptors is found in a study in which phage-displayed peptide libraries were screened for peptides that interact specifically with agonist or antagonist bound estrogen receptor (69). Many peptides isolated with estradiol-bound ER α contained the LXXLL motif, while those isolated with tamoxifen-bound receptor did not. These findings suggest that the activation of the ER α by tamoxifen that is observed in some tissues might occur via a different mechanism than estradiol-induced activation, such as through the recruitment of non-LXXLL containing coactivators to tamoxifen-specific surfaces of the ER. Crystallographic and protein structure prediction analyses have indicated that these motifs form amphipathic α -helices with the leucine residues comprising a hydrophobic surface on one face of the helix. The helix is able to interact with the AF-2 domain of the

liganded receptor via a hydrophobic groove made up of residues from receptor helices 3, 4, 5, and 12 that is the result of the conformational change induced by hormone binding (Fig. 4) (31-34, 53).

The most interesting aspect of NR box function is the revelation that a receptor-specific code exists, where different nuclear receptors prefer different NR boxes of the RID for interaction with coactivators (32, 67, 68, 70, 71). For example, a 13-aa peptide encompassing GRIP1 motif ii efficiently blocked interaction between GRIP1 and the TR β -LBD in vitro while a peptide comprising motif iii was a more potent competitor for GR binding (32). Similarly, yeast two-hybrid assays demonstrate that mutation of TIF2 motif ii is most deleterious to interactions with PPAR α while a motif i mutation has the greatest effect on the TIF2-RXR β interaction (70). In most cases, however, mutation of a single motif does not completely abolish coactivator interaction with nuclear receptors, suggesting that multiple NR boxes contribute to the overall, high-affinity binding to the receptor. It is likely that the precise arrangement of multiple motifs and structural nuances of each receptor determine the relative contribution of each NR box to the interaction.

Other determinants that contribute to NR box selectivity by different nuclear receptors include residues flanking each NR box. For instance, a chimeric peptide containing the GRIP1 NR box iii motif in the context of the flanking sequences of NR box ii competed for TR-LBD binding with a similar potency as the peptide comprising NR box ii (32). Also, using phage-displayed libraries enriched for LXXLL-containing peptides, it was demonstrated that several subclasses of these peptides exist which

contain different flanking residues and which vary in their abilities to interact with different ER mutants and other receptors (72). Furthermore, it has been shown that the flanking N-terminal amino acids are not essential, while the eight residues C-terminal to the NR box are required for SRC-1 mediated coactivation of RAR, TR, and ER (71). These studies also revealed additional preferences of ER and RAR for different NR box ii C-terminal amino acids. Intact residues +12 and +13 (where L of LXXLL is +1) are required for SRC-1 rescue of ER activity while residues at +6, +7, +11, and +13 are necessary for rescue of RAR function. Finally, because most nuclear receptors require two intact NR boxes of coactivator for interaction, the spacing between the motifs also can serve as a determinant for recognition. Deletion of 30 of the 50 amino acids between NR boxes ii and iii abolished the ability of SRC-1 to rescue IgG-mediated inhibition of RAR activity (71). In contrast, proper spacing between NR boxes i and ii was required for coactivation of PPAR γ , consistent with the requirement of intact motifs i and ii for maximal PPAR γ transactivation.

Interestingly, more recent studies have detailed a similar mechanism of interaction for the corepressors SMRT and NCoR with nuclear receptors (73-75). Both proteins contain LXXLL-like motifs, termed CoRNR boxes, in the respective receptor-interacting domains. These motifs have a consensus sequence of LXXI/HIXXXI/L and also form an α -helix. Mutation of these motifs blocked interaction with unliganded RAR or TR while peptides corresponding to these motifs were able to compete away the corepressor-receptor interaction in vitro, implicating the CoRNR box as being required for binding. In vivo, those mutations that blocked interaction with corepressor also

abolished transcriptional repression by TR. Furthermore, using receptor mutational analysis and molecular modeling, this domain appears to contact the receptor via the same hydrophobic pocket that accommodates the NR box of coactivators (73). However, due to the extended nature of the CoRNR box, the charge clamp formed by the conserved glutamate from helix 12 and the lysine from helix 3 may not be required to position this motif, but rather would inhibit corepressor binding. These observations support previous studies that demonstrate that helix 12 is not required for corepressor binding (23, 76). Overall, it suggests that the coactivator and corepressor binding sites on nuclear receptors overlap to a large degree and that helix 12 acts as the determining factor that discriminates between the binding of coactivator or corepressor in the presence or absence of ligand, respectively.

D. X-ray Crystal Structures

The biochemical studies outlined above clearly implicate the LXXLL motifs of SRC coactivators as being critical to the interaction with and coactivation of nuclear receptors. Further insight into the molecular basis of these interactions can be found in the recently solved crystal structures of several nuclear receptor LBDs with coactivator fragments containing NR boxes. In the structure of TR β -LBD complexed with T3 and a 13-aa peptide encompassing NR box ii of GRIP1 (32), the leucines of the α -helical NR box make contacts with a hydrophobic groove consisting of residues from helices 3, 4, 5, and 12 of TR β . A single LXXLL peptide interacts with each monomer of the TR β dimer.

Mutagenesis confirms the importance of these receptor residues for in vitro binding of GRIP1 to TR β . A very similar structure is observed with agonist-bound ER α -LBD complexed with a peptide comprising NR box ii of GRIP1 (Fig. 4) (31). However, in the antagonist-bound ER α -LBD structure, helix 12 of the ER α is occluding the coactivator-binding site, consistent with the inability of SRC coactivators to bind antagonist-bound nuclear receptors. Most strikingly, a region of helix 12 contains an NR box-like sequence (LXXML) and functions as an intramolecular mimic of the LXXLL motif by making contact with the hydrophobic groove. Similar results were obtained in the crystal structure of the heterodimer of the RAR α and RXR α ligand binding domains complexed with the RAR-specific antagonist BMS614 (77). The extended ring structure of the ligand prevents helix 12 from adapting the active conformation, but instead positions it in the coactivator groove where it makes contacts with residues involved in LXXLL motif binding. Therefore, the coactivator-binding site is not generated. These observations provide a molecular basis of antagonist function via conformational changes of helix 12 and inhibition of coactivator binding. The structure of the PPAR γ -LBD homodimer bound to rosiglitazone and an 88-aa fragment of SRC-1 containing NR boxes i and ii has also been described (34). This study details the function of a "charge clamp" consisting of a conserved glutamate from helix 12 and lysine from helix 3 of the LBD that positions the LXXLL motif into the hydrophobic groove of the receptor. In addition, the two NR boxes of the SRC-1 fragment are observed to contact simultaneously the PPAR γ dimer, providing further support for the role of multiple motifs in mediating coactivator-receptor interactions. Although the biological relevance of the PPAR γ homodimer is unknown,

the coactivator fragment makes similar contacts as those found in the structure of the PPAR γ /RXR α ligand binding domains crystallized with an SRC-1 peptide, 9-cis retinoic acid, and the PPAR γ ligands rosiglitazone or GI262570 (78). However, in contrast to the structures of the PPAR γ homodimer and RAR α /RXR α heterodimers, this heterodimer is asymmetrical at the dimer interface and helix 12 of PPAR γ makes contacts with helix 7 of RXR α . These differences may account for the permissive nature of PPAR/RXR heterodimers versus other RXR heterodimers. In the instances of VDR/RXR, TR/RXR, and RAR/RXR heterodimers, the hormone induced transcriptional activation function of RXR is suppressed and RXR ligand is actually prevented from binding upon heterodimer formation. Therefore, RXR is a silent partner and these heterodimers are considered "non-permissive" to RXR ligands. However, in the case of PPAR/RXR, both receptors are individually responsive, or "permissive", to their respective ligands. The additional contacts between PPAR and RXR noted above may explain these observations since helix 12 of PPAR may be stabilized by interaction with RXR in a position that permits coactivator binding even in the absence of PPAR ligand.

E. Mechanisms of Transcriptional Activation by SRC Coactivators

The SRC coactivators also contain an intrinsic transcriptional activation function, which is evident upon tethering coactivator to DNA via a heterologous DNA-binding domain. All three members are able to efficiently activate transcription when fused to the

Gal4 DNA-binding domain in both yeast and mammalian cells (48, 52, 65). Detailed deletional analysis has subsequently mapped the activation domain (AD) as being located C-terminal to the receptor-interacting domain. Interestingly, this AD also contains 3 additional LXXLL motifs representing NR boxes iv, v, and vi, that have been linked to interaction with the general transcriptional activators CBP/p300 (Fig. 3). CBP/p300 has been demonstrated to interact with SRC proteins in vitro and in vivo and mutation of one or more of the AD NR boxes markedly impairs these interactions, as well as the activation function of the coactivator (64, 71, 79). Furthermore, microinjection studies have shown that anti-CBP antibodies abolish the ability of SRC-1 to coactivate RAR, suggesting that CBP/p300 is required for the coactivation function of SRC-1 (71). However, it is worthy to note that additional, CBP-independent transcriptional activation domains have also been attributed to members of the SRC family, supporting the existence of multiple mechanisms of transcriptional activation by coactivators (41, 43, 64). The contribution of these multiple activation domains to overall coactivator function is not completely clear, but several studies suggest that coactivators may preferentially utilize specific ADs depending on the receptor or activation function (AF-1 vs. AF-2) that is mediating the response to hormone (41). In particular, the N-terminal AF-1 activation domain seems to be most critical to transcriptional activation by the androgen receptor (AR). SRC-1 and GRIP1 have been demonstrated to bind and coactivate the AF-1 domain of the AR (39-41). These interactions are mediated by the C-terminus of the SRC coactivator, rather than the centrally located domain containing NR boxes i/ii/iii. Consistent with this observation, mutation of these motifs does not inhibit the ability of

the coactivator to enhance transcriptional activation by the full-length AR, but only the isolated AF-2 domain (39, 40). This AF-1 interacting domain also lacks the CBP-interacting domain of the coactivator, thus coactivation of AF-1 likely occurs via mechanisms other than CBP recruitment, but through additional coactivators, such as CARM1 (see below), which also binds the C-terminus of GRIP1 (80). Interestingly, the N-terminus of the AR also can interact with the C-terminal AF-2 domain and is required for both AF-2 induced transcription and SRC coactivation of AF-2 activity (39). These studies suggest that the AF-1 and AF-2 domains may synergize for complete AR activity and that the interaction of the two activation domains may result in recruitment of coactivator to the AR. Finally, SRC coactivators have also been demonstrated to enhance the AF-1 activity of the ER α in the presence of both estradiol and tamoxifen, suggesting that the partial agonism of tamoxifen occurs by coactivator recruitment to the AF-1 domain (38).

Another potential mechanism of transcriptional activation by SRC coactivators is histone acetylation. Hyperacetylated histones have long been linked to transcriptionally active chromatin, for acetylation leads to an unpacking of the condensed chromatin network, thereby facilitating the access of transcription factors to target gene promoters. Accordingly, both CBP/p300 and the CBP/p300-associated factor P/CAF have been demonstrated to possess potent histone acetyltransferase (HAT) activity (81-83), which is required for transcriptional activation by CBP (84). Both factors interact with SRC coactivators, as well as with nuclear receptors themselves, and enhance receptor transcriptional activation (85-87). Therefore, recruitment of HAT-containing

coactivators by the receptor may lead to a modulation of chromatin structure, thereby facilitating the access of either additional transcriptional activators, such as the DRIP/TRAP complex (see below), or the assembly of the pre-initiation complex, ultimately leading to transcriptional activation. Interactions between coactivators and the basal transcription machinery may also play a role in transcriptional activation, for both CBP/p300 (88, 89) and SRC-1 (42, 43) have been reported to interact with TBP and TFIIB. Interestingly, moderate HAT activity has also been attributed to SRC-1 and ACTR, suggesting that liganded nuclear receptors recruit a coactivator complex containing multiple enzymatic activities (54, 90). The apparent redundancy of HAT activities among the coactivator complex remains to be resolved completely. However, transcription factor-specific differences in HAT requirements have been established for RAR versus CREB via microinjection analysis (91). Whereas P/CAF HAT activity was required for transcriptional activation by RAR, CBP HAT activity was required for CREB function. Additionally, cell-type and promoter-specific differences may also account for the existence of numerous HAT-containing coactivators. Finally, multiple HAT activities may be required if non-histone proteins also serve as substrates for these enzymes. In support of this, p300 has been demonstrated to acetylate p53, increasing its DNA binding activity (92). Also, CBP and P/CAF can acetylate TFIIE and TFIIF in vitro, which again links the basal transcription machinery to transcriptional activation by nuclear receptor coactivators (93). Furthermore, a recent study reports that ACTR itself can be acetylated by CBP/p300 (94). In this paper, hormone treatment results in enhanced histone acetylation at ER, RAR, and VDR target gene promoters and increased

recruitment of coactivators, including ACTR and CBP/p300. However, this effect is transient in nature and is strongly downregulated after prolonged hormone treatment. Surprisingly, acetylation of ACTR by CBP/p300 at specific lysine residues causes the dissociation of ACTR from the DNA-bound ER homodimer. These results suggest that the mechanism of downregulation of receptor activity involves release of the coactivator complex from the receptor via acetylation of the coactivator itself.

Finally, ligand-independent mechanisms of coactivator recruitment have also been reported. Zwijsen and colleagues demonstrated that cyclin D1 can function as a coactivator for ER α through the recruitment of SRC-1 in the absence of hormone (95). This coactivation was observed even in the context of AF-2 mutated receptors, suggesting that cyclin D1 can mediate transcriptional activation by ER α mutants that cannot bind hormone or directly recruit SRC coactivators. Interestingly, cyclin D1 bound SRC-1 directly via the domain containing the three LXXLL motifs. Peptide competition experiments revealed that NR box ii of SRC-1 was preferred by ER α while NR box iii was preferred by cyclin D1, suggesting a specificity of the NR boxes for distinct protein-protein interactions. Cyclin D1 also bound ER α directly and was required for recruitment of SRC-1 to ER α in the absence of estradiol, indicating that cyclin D1 can act as a bridging factor between ER α and SRC-1.

In another study, Tremblay, et al demonstrated that phosphorylation of the AF-1 domain of ER β led to the recruitment of SRC-1 in the absence of hormone (96). SRC-1 interacted directly with the AF-1 domain of ER β via a region that contained two consensus proline-directed kinase sites. Serine 106 of ER β was readily phosphorylated

by MAPK in vitro, which subsequently enhanced the interaction with SRC-1. In vivo, treatment of transfected cells with MAPK inhibitors impaired the interaction of SRC-1 with ER β and abolished the ability of SRC-1 to enhance transcriptional activation by the unliganded ER β , suggesting that phosphorylation-dependent recruitment of coactivator by ER β is required for maximum AF-1 activity. Interestingly, SRC-1 and AIB1 themselves can also be phosphorylated by Erk-2 (97, 98). This phosphorylation results in an enhancement of AIB1 transcriptional activation and stimulates the recruitment of p300 and its associated HAT activity (97). Taken together, these studies reveal alternative routes for coactivator recruitment to nuclear receptors that may underlie the multiple mechanisms of transcriptional activation by receptors and contribute to the specificity of receptor function.

The focus of this thesis project was a detailed analysis of RAC3 regulation of nuclear receptor function. RAC3 is a polymorphic variant of ACTR/AIB-1 that was independently isolated in our laboratory (52). It was demonstrated that the LXXLL motifs of RAC3 are key contributors to RAC3 interactions with nuclear receptors, in particular VDR, ER β , and RAR α , and also the general transcriptional coactivator CBP. Specifically, receptor-specific preferences of these nuclear receptors for the three NR boxes of the RAC3 receptor-interacting domain were uncovered. These motifs differentially regulate interactions between the coactivator and each receptor both in solution and when bound to DNA. Consequently, they also differentially participate in RAC3 enhancement of transcriptional activation in vivo. Furthermore, the NR boxes of the RAC3 activation domain, particularly NR box v, are critical to coactivator interaction

with CBP. This recruitment of CBP was found to be the major mechanism of RAC3 coactivation of RAR α activity, but apparently was dispensable for stimulation of ER β activity. This finding suggested that RAC3 can activate transcription by multiple mechanisms. Finally, the cofactor-binding pocket of RAR α and RXR α was also characterized. Recent structural data have demonstrated that this hydrophobic pocket is comprised of helices 3, 4, 5, and 12 of the receptor ligand-binding domain. This work reveals that these intact helices are required for RAC3 recruitment to liganded retinoid receptors in vitro, and therefore, also required for transcriptional activation by the receptor in vivo. Thus, coactivator recruitment to the receptor is essential for transactivation. Interestingly, helices 3, 4, and 5 are also required for unliganded receptor interactions with the corepressor SMRT and therefore, transcriptional repression. These findings indicate that the coactivator and corepressor binding pockets of retinoid receptors overlap to a great extent and that helix 12 plays a key regulatory role in cofactor binding to the receptor. Overall, this project has uncovered several novel points concerning RAC3 regulation of nuclear receptor function and has contributed significantly to the field of transcriptional regulation by these receptors and cofactors.

CHAPTER II

THESIS GOALS AND SPECIFIC AIMS

In the past few years, the field of nuclear receptor research has witnessed an explosion in data, be it biochemical, genetic, or crystallographic, concerning the mechanisms of transcriptional regulation by steroid/nuclear receptors. While the biological responses to hormones have been studied for quite some time, it has only been in recent times that we are beginning to understand the cellular factors that mediate these responses. It has become clear that nuclear receptors recruit cofactors termed corepressors and coactivators in order to regulate the expression of their respective target genes. In the case of coactivators, much work has been done in detailing the mechanisms by which the first known nuclear receptor coactivator, SRC-1, regulates receptor function. These studies include the discovery of the LXXLL motif or NR box, an α -helical domain that has been demonstrated to be critical to the ability of SRC-1 to interact with liganded receptors and activate transcription. However, little is known about the regulation of receptor function, particularly of RAR α , VDR, or ER β , by RAC3, the third member of the SRC family of coactivators that was previously cloned in our laboratory. Therefore, the major goal of my thesis research was to characterize in detail the mechanisms by which the coactivator RAC3 modulates the activity of these members of the steroid/thyroid hormone receptor superfamily. The specific aims of the project were as follows.

The first aim of the project was to characterize the mechanisms by which RAC3 regulates the activity of nuclear receptors. In particular, the role of the LXXLL motifs of the RAC3 receptor-interacting domain (NR boxes i-iii) were investigated in order to determine if different nuclear receptors preferred different NR boxes for interaction with RAC3. To accomplish this, site-directed mutagenesis and peptide-competition experiments were performed to map RAC3 interactions with nuclear receptors *in vitro*. Further insight into these interactions was gained by studying the recruitment of RAC3 to DNA-bound nuclear receptors. In order to provide functional evidence for RAC3 regulation of receptor function *in vivo*, the ability of RAC3 to enhance transcriptional activation by these receptors and the NR box requirements of this coactivation activity were also determined.

Since RAC3 contains a domain that is able to activate reporter expression when fused to the Gal4 DNA-binding domain, the transcriptional activation function of the coactivator was also characterized in the second aim of the project. The role that the C-terminal LXXLL motifs (NR boxes iv-vi), which are found in the RAC3 activation domain, play in this activation were determined by site-directed mutagenesis. Furthermore, the mechanism of transcriptional activation was studied by analyzing RAC3 interactions with the general transcriptional cointegrator CBP/p300. Finally, the contribution of CBP/p300 interaction to the coactivation of nuclear receptor activity by RAC3 was assayed *in vivo* by transient transfection.

The third aim of this work investigated the receptor requirements for interaction with the coactivator RAC3, as well as with the corepressor SMRT. Based on structural

studies, it has been hypothesized that both coactivators and corepressors interact with a hydrophobic pocket in the receptor ligand-binding domain that consists of helices 3, 4, 5, and 12. To test this, the cofactor-binding pocket of retinoid receptors was characterized. The effects of mutation of the above α -helices on receptor interactions with RAC3 and SMRT were determined in vitro. In addition, the relative contribution of the cofactor-binding pocket of each monomer in a DNA-bound receptor dimer to cofactor recruitment was examined. These analyses were then correlated with the transcriptional activity of retinoid receptors in vivo.

It is the goal of this project to contribute significantly to the understanding of steroid/nuclear receptor regulation of gene expression. Although receptor function has been studied intensely in recent years, the precise mechanisms by which hormone action is mediated are still not clear. Therefore, during my thesis research, I attempted to gain insights into the manner in which steroid/nuclear receptors are modulated by the coactivator RAC3 and the corepressor SMRT. If we understand the details of cofactor recruitment to receptors and how, in turn, the transcriptional activity of the receptor is regulated, it will allow us to better comprehend the overall pathway that begins with the arrival of hormone at a target cell and ends with the physiological response that is elicited by the body.

CHAPTER III

EXPERIMENTAL PROCEDURES

A. Recombinant Protein Expression and Purification

Plasmids encoding GST fusion proteins were transformed into *E. coli* BL21 competent cells and subsequently grown up in 100 mL LB broth containing 100 µg/mL ampicillin to OD₆₀₀=0.4-0.55. Protein expression was then induced for 3-4 hours with 1mM IPTG and cells were harvested and washed in 10 mM Tris, pH=8/150 mM NaCl/1 mM EDTA (STE). Cells were then resuspended in STE, and 5 mM DTT, 1 mM PMSF, and 1.5% Sarkosyl were added. Cells were sonicated and centrifuged at 8000 rpm for 30 minutes to remove cell debris. To the supernatant, 2% Triton X-100 and 100 µL of a 50% slurry of Glutathione-sepharose beads were added and rotated for 30 minutes at 4°. The beads were then collected by centrifugation and washed 4-5 times with cold PBS before final resuspension in storage buffer containing 50 mM Hepes/150 mM NaCl, 5 mM DTT, and 10% glycerol.

B. Far-Western Analysis

Purified GST fusion proteins were separated by SDS-PAGE and electroblotted onto a nitrocellulose membrane. After blocking overnight at 4° in HBB (25 mM Hepes-KOH, pH=7.7/25 mM NaCl/5 mM MgCl₂) plus 5% nonfat milk/1 mM DTT/0.05% NP-40,

proteins were denatured with 6 M guanidine hydrochloride (GnHCl) in HBB and subsequently renatured in stepwise dilution of GnHCl at concentrations of 3 M, 1.5 M, 0.75 M, 0.375 M, and 0.187 M. Membranes were then blocked again for 1 hour at room temperature in the above blocking buffer followed by 30 minutes at room temperature in the above buffer substituted with 1% nonfat milk and 0.1 mM methionine. Membranes were subsequently hybridized overnight at 4° with ³⁵S-methionine labelled protein diluted in hybridization buffer (20 mM Hepes, 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl₂, 0.05% NP-40, 1 mM DTT, 0.1 mM methionine). The membrane was washed three times at room temperature in hybridization buffer and bound probe detected by autoradiography. ³⁵S-labelled probes were generated by Quick-coupled in vitro transcription/translation (Promega). For peptide competition experiments, the given concentration of peptide was added to the probe 10 minutes prior to hybridization with the membrane. Peptides were synthesized by the University of Massachusetts Medical School peptide facility with sequences as follows: NR box i peptide (LESKGHKLLQLLTLSSDDRGHSSL), NR box ii peptide (LQEKHRILHKLLQNGNSP), NR box iii peptide (KKKENNALLRYLLDRDD), control peptide (GSGSATATLYENKPRPPYIL). Radioactive bands were quantified by PhosphorImager using the ImageQuant software (Molecular Dynamics). Far-Western experiments were repeated three times and peptide competition data are representative of duplicate experiments.

C. GST pull-down assay

Approximately 5 μ g of purified GST fusion protein was incubated with 5 μ L of 35 S-methionine labelled protein with moderate shaking at 4 $^{\circ}$ overnight in binding buffer (20 mM HEPES/pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl₂, 0.05% NP40, 1 mM DTT, 1 mg/mL BSA). The bound protein was washed three times with binding buffer and beads collected by centrifugation. The bound protein was eluted in SDS sample buffer, subjected to SDS-PAGE, and detected by autoradiography. GST pull-down experiments were repeated at least twice.

D. Site-directed mutagenesis

NR box mutants were generated with the Quick-change site-directed mutagenesis system (Stratagene). Mutagenesis utilizes a supercoiled, double stranded DNA template with the insert of interest and two, synthetic, complementary oligonucleotide primers containing the desired mutation. The primers extend during temperature cycling by means of *PfuTurbo* DNA polymerase. Then, the parental DNA template is digested away with DpnI, which is specific for methylated DNA, thus selecting for mutation-containing synthesized DNA. The sequences of all mutant constructs were confirmed by dideoxynucleotide chain-termination reactions using the T7 Sequenase protocol (USB).

E. Gel electrophoresis mobility shift assay

The sequence of the DR3 element used for VDR/RXR gel shift assays is AGCTTAAGAGGTCAGAAAGGTCACCTCGCAT. The sequence of the DR5 element used for RAR/RXR assays is AGCTTAAGAGGTCACCGAAAGGTCACCTCGCAT. The sequence of the DR1 element for RXR/RXR assays is AGCTTAAGAGGTCAAAGGTCACCTCGCAT. The double-stranded oligonucleotide probe was end-labeled with ^{32}P -dCTP by standard Klenow fill-in reaction. Unincorporated nucleotides were subsequently removed by purifying the probe with the Micro Bio-Spin P-30 column (Bio-Rad). The purified probe was incubated with in vitro transcribed/translated receptors in binding buffer containing 7.5% glycerol, 20 mM HEPES/pH 7.5, 2 mM DTT, 0.1%NP40, 1 μg poly-dIdC and 100 mM KCl. Wild-type or mutant GST-RAC3-RID was eluted from glutathione agarose beads with 10 mM reduced glutathione and added to the binding reaction. The DNA-protein complex was formed on ice for one hour and resolved on a 5% native polyacrylamide gel, which was subsequently dried and subjected to autoradiography. Experiments were repeated at least twice with a representative result shown.

F. Cell Culture and Transient Transfection

HEK293 and CV-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 5 $\mu\text{g}/\mu\text{L}$ gentamycin at 37°/5%CO₂.

Cells were plated for transfection in DMEM supplemented with 10% resin-charcoal stripped fetal bovine serum in 12- or 6-well plates one day prior to transfection. HEK293 cells were transfected using the standard calcium phosphate method while CV-1 cells were transfected using Lipofectamine according to manufacturer's protocol (Gibco.) Twelve hours after transfection, cells were washed with PBS and refed fresh media containing the indicated concentration of ligand. After 24 hours, cells were harvested for β -galactosidase and luciferase activities in lysis buffer containing 25 mM Tris- $\text{PO}_4/\text{pH}=7.8$, 15% glycerol, 2% CHAPS, 1% Lecithin, 1% BSA, 4 mM EGTA, 8 mM MgCl_2 , 1 mM DTT, and 0.4 mM PMSF as described (76). Luciferase activity was determined with a MLX plate luminometer (Dynex) using 50 μL of cell lysate and 100 μL each of buffer A (0.1 M KPO_4 /5 mM ATP/10 mM MgCl_2) and buffer B (0.09 M KPO_4 /1 mM Luciferin). Luciferase activity was then normalized to β -galactosidase activity in order to account for differences in transfection efficiency between samples. Transient transfections were performed in triplicate and each experiment was repeated at least twice.

CHAPTER IV

RESULTS

CHAPTER IV-1

DIFFERENTIAL UTILIZATION OF RAC3 LXXLL MOTIFS BY RECEPTORS

A. VDR and ER β interact with multiple surfaces of RAC3

Our laboratory has previously defined the minimal receptor interacting domain (RID) of RAC3 to be amino acids 613-752, which contains the first three LXXLL motifs (52). I wished to further determine if different receptors were capable of binding to the same regions of RAC3. To accomplish this, a panel of GST-RAC3 fusion proteins, in total comprising the full-length RAC3, was purified and probed with ^{35}S -methionine labeled VDR and ER β in a Far-Western assay (Fig. 5). The VDR, as expected, interacted in a ligand-dependent manner with GST-RAC3 613-752 in this assay (Fig. 5B). It also bound GST-RAC3 723-1034, which only contains NR box iii, in a ligand-dependent manner. No interactions were evident in the absence of vitamin D. The VDR did not interact with any other GST-RAC3 fragment, including GST-RAC3 342-646, which contains NR box i. It also appeared that the VDR interacted more strongly with GST-RAC3 723-1034 than with GST-RAC3 613-752, suggesting a more important role for NR box iii in the RAC3-VDR interaction. However, a different pattern was evident upon repeating this assay with ^{35}S -ER β , for in addition to ligand-dependent interactions with GST-RAC3 613-752 and 723-1034, ER β also bound the 342-646 fragment, which contains only NR box i (Fig. 5C). These interactions were of approximately equal

intensity. There was also a weak, ligand-independent interaction with GST-RAC3 1-407. Identical results were obtained for ER α (data not shown). A Coomassie Blue-stained polyacrylamide gel of the GST-RAC3 fusion protein confirmed the identity of each GST-RAC3 fusion protein and approximately equal protein concentrations in each lane (data not shown). Therefore, the VDR and ER β display different binding patterns for RAC3 fragments, with the VDR interacting preferentially with regions containing NR box iii and the ER β interacting equally well with regions containing any of the three NR boxes and the N-terminal bHLH-PAS domain.

B. NR Box peptides differentially compete with nuclear receptors for RAC3 binding

We then wanted to investigate the relative importance of individual NR boxes within the RAC3-RID in mediating the interactions between RAC3 and the VDR or ER β . Peptides were synthesized corresponding to NR boxes i, ii, and iii, which were then tested for their relative abilities to compete with the RAC3-RID for VDR or ER β binding. To do this, peptide at concentrations of 0, 2.5, 5, 12.5, or 25 μ M were incubated with 35 S-labeled receptor and 1 μ M ligand prior to probing the GST-RAC3 613-752 fragment in the Far-Western assay. With the VDR, peptides corresponding to the second and third LXXLL motif were able to compete away the RAC3-RID interaction with VDR in a dose-dependent manner (Fig. 6A). However, peptide iii was much more potent, for as little as 2.5 μ M peptide abolished nearly all of the GST-RID interaction with 35 S-VDR. This observation was confirmed upon quantifying the data, for the IC₅₀ of peptide iii was

several fold lower than that of peptide ii (Fig. 6B). The peptide comprising NR box i had little, if any, effect on the VDR-RID interaction. A control experiment demonstrated that the effects of these peptides were specific, for a random peptide did not alter the interaction between the ^{35}S -VDR and GST-RAC3-RID (Fig. 6C). We again identified a different pattern when the same experiment was done with the ER β (Fig. 7). Here, all three peptides were able to compete efficiently for ER β binding with the RAC3-RID, with peptide ii being the most potent. Thus, these data reveal receptor-specific preferences for interactions between RAC3 and different nuclear receptors, for the VDR and ER β have different affinities for the NR boxes of RAC3. Residues flanking each LXXLL motif likely contribute to this specificity, for the motifs themselves are so highly conserved that surrounding amino acids may influence the affinity of a particular receptor for the particular NR box.

C. NR box mutations can impair RAC3 interactions with nuclear receptors in vitro

In order to assess the integrity of the LXXLL motif itself in mediating the interaction between the RAC3-RID and nuclear receptors, we used site-directed mutagenesis to switch the leucine residues of each motif to alanines (Fig. 8A). The mutants were made using the GST-RAC3-RID fusion as the template, confirmed by DNA sequencing and tested for their ability to interact with the ^{35}S -VDR, ^{35}S -RAR α , ^{35}S -RXR α or ^{35}S -ER β in GST-pulldown assays (Fig. 8B). The wild-type GST-RAC3-RID was able to pull down a significant amount of ^{35}S -VDR in the presence of 1 μM Vitamin

D. This interaction was specific, for GST alone pulled down much less ^{35}S -VDR. Mutations in NR boxes i or ii displayed wild-type binding. However, when NR box iii was mutated, the RID-VDR interaction was greatly reduced to a level only slightly higher than background binding to GST alone. GST-RAC3 342-646, which contains only NR box i, also had minimal binding to the VDR, consistent with the Far-Western assay. Equal protein concentrations of each GST fusion confirmed the specificity of these findings (data not shown). Thus, these data support the above observations in implicating NR box iii as being most critical to RAC3 interaction with the VDR in vitro.

We also assessed the NR box requirements of retinoid receptors. Wild-type GST-RAC3 RID efficiently precipitated RAR α in the presence of 1 μM all-trans retinoic acid. In contrast to the VDR, mutation of NR box iii had little effect on this interaction. However, mutation of NR box i reduced binding significantly while the NR box ii mutation abolished the RAR α -RID interaction altogether. These observations imply that RAC3 NR box ii is most important for interactions with RAR α . Similar results were also obtained with RXR α , with NR boxes i and ii mediating the interaction with the RAC3-RID. However, unlike RAR α , mutation of either NR box i or ii had about the same effect on the interaction, reducing it to only slightly above background binding. Therefore, motif i may be more important to coactivator interactions with RXR α while motif ii is critical to interactions with RAR α .

Finally, the wild-type RID was also able to pull down significant amounts of ^{35}S -ER β in the GST-pulldown assay while the GST only control demonstrated minimal interaction. In contrast to the other receptors, alanine substitution for leucine in any of

the three NR boxes weakened the interaction of the RAC3-RID with ER β , with the mutation of NR box ii being the most deleterious, again supporting the results of LXXLL peptide competition experiments. However, with each mutation, significant binding above background between the RAC3-RID and ^{35}S -ER β was still observed. Furthermore, the GST-RAC3 342-646 fragment, with only NR box i, was able to interact efficiently with ER β as in the Far-Western assay. These data suggest that although all three motifs are capable of interacting with ER β separately, none of them is absolutely required for the interaction. In contrast, NR box iii of RAC3 appears to be essential for the interaction with the VDR and NR box ii essential for interaction with RAR α . These subtle, but precise differences in NR box requirements also suggest potential differences in the coactivator binding pocket on each receptor.

D. RAC3-RID interactions with DNA-bound nuclear receptors

The above data provide compelling evidence that the VDR and RAR α interact specifically with RAC3 in solution via the LXXLL motifs of the RAC3 RID, particularly NR box iii for VDR and NR box ii for RAR α . To gain further insight into the function of NR boxes in coactivator-receptor interactions on a heterodimeric complex bound to DNA, we performed gel-shift assays with VDR/RXR and RAR/RXR heterodimers on a DR3 or DR5 element, respectively, in the presence of wild-type or mutant RAC3-RID. The DR3 element consists of two half-sites, in a direct repeat, with the sequence AGGTCA. These two half-sites are separated by 3 nucleotides, which makes this

element specific for the VDR/RXR heterodimer versus other RXR heterodimers. The DR5 element contains the same half-site, but separated by 5 nucleotides. These probes were labeled with ^{32}P -dCTP using the Klenow fragment of DNA polymerase and incubated with in vitro transcribed/translated receptors and the indicated GST fusion protein, which was eluted from the glutathione-sepharose beads with excess glutathione. The VDR/RXR heterodimer bound strongly to the ^{32}P -labelled DR3 probe and was unaffected by addition of GST alone (Fig. 9, lanes 1,2). Addition of the RAC3-RID resulted in a shift of the heterodimeric complex to a slower migrating form only in the presence of 1 μM vitamin D (arrow, lanes 3 and 4), consistent with the ligand-dependent interaction between the RAC3-RID observed in the Far-western assay. Mutation of NR box i had little effect on the ability of the RID to shift the complex (lane 5). However, mutating NR box ii diminished the formation of the RID-VDR/RXR complex, while mutating NR box iii nearly abolished the formation completely (lanes 6 and 7). Consistently, GST-RAC3 342-646, with only NR box i, was unable to shift the VDR/RXR complex (lane 8). These results differ slightly from the GST- pulldown, in which only the NR box iii mutation inhibited interaction with VDR alone. However, they are consistent with the hypothesis that suggests that two motifs may contribute to the interaction with DNA-bound nuclear receptor heterodimers, with one LXXLL motif per monomer.

Many studies have demonstrated that helix 12 of nuclear receptors, which comprises the ligand-dependent AF-2 domain, is critical to coactivator binding (99). As detailed above, it is believed that high-affinity interactions between receptors and SRC

coactivators requires a ligand-induced repositioning of this helix. However, the role of each helix 12 of a DNA-bound nuclear receptor heterodimer in recruiting coactivators is not completely understood. In order to gain insight into this, we analyzed the involvement of nuclear receptor AF-2 domains in regulating the interaction between the RAC3-RID and the VDR/RXR heterodimer. Using the gel-shift assay, we compared the ability of the RID to bind the wild-type heterodimer versus the VDR/RXR443 and VDR402/RXR heterodimers, in which the AF-2 domain of RXR or VDR had been deleted, respectively (Fig. 10). As demonstrated above, the RID was able to bind the DNA-bound, wild-type VDR/RXR complex (Fig. 10, lane 2). This shift appears weaker than that in the previous experiment due to underexposure of the autoradiograph in order to clearly see the other bands on the gel. Deletion of the VDR AF-2 domain did not affect the formation of the heterodimer-DNA complex (lane 3), but resulted in the loss of the RID-shifted complex (lane 4). This suggests that the VDR AF-2 domain is required for interaction of RAC3 with the heterodimer, and that RXR AF-2 domain alone is not sufficient for the interaction. Interestingly, deletion of the RXR AF-2 domain resulted in a much stronger shift of the heterodimeric complex by the RAC3-RID (lane 6) without affecting heterodimer formation (lane 5), suggesting that the RXR AF-2 domain can inhibit the interaction between RAC3 and VDR/RXR. The strong interaction was abolished upon deletion of the VDR AF-2 domain (lane 8), further supporting a requirement of VDR AF-2 helix 12 for RAC3 binding to the DNA-bound heterodimer. These findings are consistent with a model of allosteric inhibition of coactivator binding to the RAR/RXR heterodimer by the RXR AF-2 domain (100).

Additionally, we compared the RAC3 NR box preferences of VDR/RXR versus VDR/RXR443 (Fig. 11A). Intriguingly, the VDR/RXR443 heterodimer displayed different NR box preferences. Mutation of NR box i or iii greatly reduced the shift by the RAC3-RID (lanes 3 and 5), while mutation of NR box ii only slightly weakened the binding (lane 4). Thus deletion of the RXR AF-2 domain resulted in a switch in the NR box requirements, with NR boxes i and iii being most important for VDR/RXR443 compared to NR boxes ii and iii for VDR/RXR. An autoradiograph confirmed equal expression levels of each ^{35}S -labeled receptor (Fig. 11B). This finding supports the hypothesis that multiple LXXLL motifs provide RAC3 with the flexibility to adapt to different configurations of a nuclear receptor dimer. It also suggests that the RXR AF-2 domain may be involved in the positioning of specific NR boxes within the VDR/RXR heterodimer.

In the analysis of $\text{RAR}\alpha$ - $\text{RXR}\alpha$, the heterodimer specifically bound to the ^{32}P -labeled DR5 probe in the presence of all-trans retinoic acid (Fig. 12, lane 2). Addition of GST alone had no effect on the complex while the wild-type RAC3-RID shifted a portion of the complex to a slower migrating form (lanes 3,4). However, mutating NR box i of the RAC3-RID inhibited somewhat the interaction with $\text{RAR}\alpha$ - $\text{RXR}\alpha$, while mutating NR box ii abolished it completely (lanes 5,6). Mutation of NR box iii had no effect on the RID-induced shift (lane 7). These observations correlate completely with the results of the GST pulldown experiments. Thus, the $\text{RAR}\alpha$ - $\text{RXR}\alpha$ heterodimer has different NR box requirements than the VDR- $\text{RXR}\alpha$ heterodimer, which required an intact NR box ii and iii for interaction with the RAC3-RID. In analyzing the role of helix 12 of

each receptor, it was evident that, similar to VDR-RXR α , helix 12 of RXR α inhibited the interaction with RAC3, for deletion of this helix resulted in a complete shift of the RAR α -RXR α complex (lane 9). Conversely, helix 12 of RAR α is absolutely required for coactivator interaction, for deletion of helix 12 of RAR α blocks the interaction with the RAC3-RID (lane 11). In summary, it is evident that the different RXR heterodimers have different RAC3 NR box preferences for coactivator binding, but share the requirement of helix 12 of the RXR partner and are inhibited by helix 12 of RXR itself.

Finally, we determined the NR box requirements of the RXR α /RXR α homodimer, which is able to bind at a DR1 response element (Fig. 13). No interaction with RAC3 was evident in the absence of 9-cis retinoic acid or upon addition of purified GST alone (lanes 2,3). However, the wild-type RID specifically shifted a portion of the homodimer to a slower migrating form in a ligand-dependent manner (lane 4). Mutation of NR boxes i or ii completely abolished this interaction, while mutation of NR box iii had minimal effect, consistent with the GST pulldown data (Fig. 8).

E. Effects of NR box mutations on RAC3 coactivation function in vivo

RAC3 has previously been shown to enhance the transcriptional activity of the retinoic acid receptor (RAR) and progesterone receptor (PR) (52). However, its effect on VDR and ER β function in vivo has not been demonstrated. To address this, we performed transient transfection assays in HEK293 and CV-1 cells using luciferase reporters harboring either two copies of the VDRE of the osteopontin gene for VDR

studies or a consensus ERE element for ER β studies (Fig. 14). The reporter construct was transfected into cells with expression plasmids for the receptor, wild-type or mutant RAC3, and beta-galactosidase. Cells were treated with hormone or solvent control and subsequently harvested. Luciferase activity was then measured and normalized to beta-galactosidase activity, thus accounting for differences in transfection efficiency between samples. Transfection of the VDR into CV-1 cells minimally activated the VDRE-driven reporter. However, treating these cells with 40 nM vitamin D strongly stimulated its activity. Cotransfection of wild-type RAC3 further enhanced VDR transcriptional activation by approximately 50%, consistent with the coactivation function of RAC3 for RAR and PR (Fig. 14A).

We then analyzed the role of the NR boxes in mediating the ability of RAC3 to potentiate VDR activity (Fig. 14A). Mutations of each NR box of the RAC3-RID were made in the context of the full-length RAC3 expression plasmid and confirmed by DNA sequencing. They were then tested for their ability to coactivate the VDR in transient transfection assays. Mutation of NR box i did not inhibit RAC3 enhancement of VDR transactivation, consistent with its inability to block the interaction of RAC3 with VDR in vitro. However, the RAC3 NR box ii or iii mutations abolished the function of RAC3 in enhancing VDR activity, reducing VDR activity to that observed in the absence of transfected RAC3. Therefore, these in vivo data are consistent with the above in vitro gel-shift data demonstrating that NR boxes ii and iii of RAC3 are critical to VDR interaction in the context of the DNA-bound VDR/RXR heterodimer. Taken together, it

is clear that NR boxes ii and iii are both involved in RAC3 regulation of VDR/RXR function, while the role of NR box i appears minimal.

We then repeated these experiments with the ER β (Fig. 14B). Estradiol treatment of CV-1 cells transfected with the ER β and the ERE-Luciferase reporter activated reporter expression approximately 8-fold. Cotransfection of wild-type RAC3 resulted in a strong, 6-fold enhancement of ER β activity. It is evident from these data that RAC3 is a more potent coactivator for the ER β than for the VDR. Cotransfection of RAC3 expression plasmids containing mutations in NR boxes i, ii, or iii all suppressed the ability of RAC3 to coactivate the ER β , with the NR box ii mutant having the greatest and NR box iii mutant having more modest effects on RAC3 function. However, all three mutant RAC3 constructs still retained some coactivation function, consistent with the ability of the GST-RAC3-RID protein harboring a single NR box mutation to still bind ER β in vitro. Thus, these in vivo data correlate with the in vitro data in implicating all three NR boxes of the RAC3-RID as being involved in RAC3 regulation of ER β function, with none of the motifs being absolutely required. These data suggest that ER β has greater flexibility than VDR in selecting different RAC3 NR boxes.

Finally, we decided to assess the functional consequences of the antagonism of RAC3 interaction with DNA-bound VDR/RXR by the RXR AF-2 domain observed in gel-shift assays. To do this, we compared the ability of RAC3 to coactivate VDR/RXR versus VDR/RXR443 activities by transient transfection assays (Fig. 15). When wild-type VDR and RXR were expressed in HEK293 cells, RAC3 was able to enhance transcriptional activation of the VDRE-driven reporter by approximately 50% (left).

However, when VDR was coexpressed with RXR443, RAC3 displayed a 2.5-fold enhancement of receptor activity, likely via increased interaction with RAC3 (center). As expected, the VDR402 mutant was transcriptionally inactive and RAC3 could not modulate its activity (right). Thus, these *in vivo* data are consistent with the gel-shift data in demonstrating that the RXR AF-2 domain can inhibit RAC3 modulation of the VDR/RXR heterodimer while the VDR AF-2 domain is absolutely required for this regulation.

Overall, the above data clearly reveal receptor-specific preferences for the α -helical NR boxes of the RAC3 receptor-interacting domain. Motifs ii or iii were absolutely essential to RAC3 interactions with the VDR or RAR α , respectively, while motifs i and ii made equal contributions to interaction with RXR α . Interestingly, all three NR boxes appeared to be involved in interactions with ER β , but none were individually required for this interaction. This suggests that coactivator interactions with the steroid receptors such as ER β may be more flexible than those with non-steroid receptors. Furthermore, two motifs were essential to RAC3 recruitment to a DNA-bound VDR/RXR or RAR/RXR heterodimer, which is consistent with the hypothesis that a single coactivator molecule binds to a receptor dimer using one NR box per monomer. Finally, the same NR boxes that mediated interactions with receptors *in vitro* were also demonstrated to mediate RAC3 enhancement of transcriptional activation by these receptors *in vivo*.

CHAPTER IV-2

CHARACTERIZATION OF RAC3 TRANSACTIVATION FUNCTION

One mechanism by which nuclear receptor coactivators activate transcription may be via recruitment of the general transcriptional activators CBP/p300 (53, 64, 85, 101). Our laboratory has previously demonstrated that RAC3 is able to interact with two fragments of CBP in vitro, CBP-C (1921-2120) and CBP-D (2041-2240) (57). Interestingly, RAC3 contains three additional LXXLL motifs, termed NR boxes iv, v, and vi, in the previously defined activation domain (Fig. 3). In light of these findings, we decided to determine if these NR boxes mediated the interaction with CBP and were involved in transcriptional activation by RAC3 in vivo. NR boxes iv, v, and vi were mutated by leucine to alanine substitution via site-directed mutagenesis (Fig. 16A). The template for this mutagenesis was a Gal4-DNA-binding domain fusion to RAC3 401-1204, which contains the functional regions of RAC3. The wild-type or mutant constructs were cotransfected into HEK293 cells with a reporter containing four tandem copies of Gal4-DBD binding sites. Wild-type Gal-RAC3 401-1204 activated this reporter 3-fold compared to the activity of the Gal4-DBD alone (Fig. 16B). Mutation of NR boxes iv or vi had little effect on the ability of Gal-RAC3 401-1204 to activate transcription. However, mutation of NR box v completely inhibited this transcriptional activation function, reducing the activation level to the basal level of the Gal4-DBD alone. To determine if this observation was a result of the inability of RAC3 401-1204

(mut v) to bind CBP, we labeled each Gal4-RAC3 401-1204 construct with ^{35}S -methionine by in vitro transcription/translation and probed the RAC3-interacting fragments of CBP in a Far-western assay (Fig. 17). Each construct expressed at an equal level in the transcription/translation reaction and, consistent with its lack of transactivation function, mutation of NR box v drastically reduced RAC3 401-1204 binding to both CBP-C and CBP-D fragments (Fig. 17A). Upon quantifying the data, it was determined that Gal-RAC3 401-1204(mut v) displayed a 50- and 100-fold lower affinity for CBP-C and CBP-D, respectively, compared to the wild-type probe (Fig. 17B). Mutation of NR boxes iv or vi had minimal effects on CBP interaction. Therefore, these in vitro and in vivo data correlate perfectly in implicating NR box v of RAC3 as being critical to transcriptional activation through CBP recruitment.

In order to determine the role of CBP recruitment in RAC3 enhancement of nuclear receptor transcriptional activation, we tested the ability of these mutants to coactivate both RAR α and ER β by transient transfection assays (Fig. 18). Each activation domain mutant was first subcloned into the full-length RAC3 expression vector that lacks the Gal4-DBD fusion. The wild-type or mutant RAC3 was then cotransfected into 293 cells with receptor expression plasmids and RARE- or ERE-driven luciferase reporter constructs. In the RAR α analysis, wild-type RAC3 enhanced RAR α activity by 85% in the presence of 10 nM all-trans retinoic acid (Fig. 18A). Mutation of RAC3 NR boxes iv or vi had no effect on this stimulation of transcription. However, mutation of NR box v did slightly reduce the ability of RAC3 to coactivate RAR α , consistent with the above results that demonstrate that mutation of RAC3 NR box v

reduces its intrinsic transactivation function and reduces CBP binding. Nevertheless, it is worthy to note that RAC3-mut v still retained the capacity to enhance RAR α activity, suggesting a CBP-independent mechanism of activation by the coactivator.

Surprisingly, a much different result was observed when the same experiment was done with ER β (Fig. 18B). Wild-type RAC3 again strongly enhanced estrogen receptor activity in the presence of 15 nM estradiol. However, unlike RAR α , mutation of neither NR boxes iv, v, nor vi had any inhibitory effect on the ability of RAC3 to coactivate ER β . This indicates that CBP recruitment is dispensable for RAC3 enhancement of ER β activity and suggests that other mechanisms of RAC3 transcriptional activation, such as interaction with CARM1, the DRIP/TRAP complex, or the general transcription machinery also contribute to RAC3 transcriptional activation.

Finally, we wished to compare the coactivation function of RAC3 with that of the other two members of the SRC family of coactivators, namely SRC-1 and TIF2, in order to determine if functional differences existed between these proteins (Fig. 19). For ER β , RAC3 potently enhanced estradiol-induced transcriptional activation several fold, consistent with the above data. Intriguingly, transfection of TIF2 resulted in a similar enhancement of ER β activity, while SRC-1 appeared to have no effect on receptor function in our hands (Fig. 19A). This experiment was also performed with the RAR α (Fig. 19B). Here, only RAC3 was capable of stimulating RAR α activity in the presence of retinoic acid, demonstrating about a 70% increase in activity. These findings suggest that SRC coactivators possess receptor-specific coactivation functions, with RAC3 possibly being a more flexible coactivator for multiple nuclear receptors.

In this chapter, it has been demonstrated that the C-terminal NR boxes, particularly NR box v, are critical to the intrinsic transcriptional activation function of RAC3. Mutation of this motif inhibited the ability of a Gal4-DNA binding domain fusion of RAC3 to enhance the expression of a reporter containing Gal4 binding sites. This effect correlated with the inability of RAC3 (mut v) to bind the general transcriptional activator, CBP, thereby elucidating a mechanism of activation by RAC3. However, other mechanisms do clearly exist, for this mutant was still able to strongly potentiate transcription by the ER β . RAR α coactivation was slightly reduced by expression of RAC3 (mut v), thus CBP binding may contribute to RAC3 enhancement of RAR α activity. Additionally, it was evident that functional differences exist between the members of the SRC family of coactivators, for RAC3 and TIF2, but not SRC-1 were able to enhance ER β activity while only RAC3 could coactivate RAR α .

CHAPTER IV-3

THE COFACTOR BINDING POCKET OF RETINOID RECEPTORS

A. Receptor residues required for cofactor interaction in vitro

It has been clear for some time that the AF-2 domain of nuclear receptors undergoes a drastic conformational change upon ligand binding that facilitates the binding of coactivators (102). This change is characterized by a folding back of helix 12 of AF-2 in which it packs against the LBD and is absolutely required for coactivator interaction. More recent crystallographic evidence has detailed the formation of a hydrophobic groove consisting of residues from helices 3, 4, 5, and 12 that accommodates the α -helical LXXLL motif of SRC coactivators (31-34). In addition, a charge clamp consisting of a highly conserved glutamate from helix 12 and a conserved lysine from helix 3 serves to position the LXXLL motif in this hydrophobic groove, based on the structure of the PPAR γ homodimer with an SRC-1 fragment containing two LXXLL motifs (34). In light of these findings, we decided to investigate the involvement of specific residues within these critical helices in cofactor binding by retinoid receptors. Upon aligning several nuclear receptor LBD sequences, highly conserved residues that are part of the cofactor binding pocket and contact the LXXLL motif based on structural data were selected and mutated by site-directed point mutation in the context of a full-length RAR α expression vector (Fig. 20). The mutations were: V240R from helix 3,

F249R from helix 4, L261R from helix 5, and E412K, which represents the charge clamp glutamate in helix 12. Each RAR α construct was then labeled with ^{35}S -methionine by in vitro transcription/translation reaction. Wild-type and mutant receptors expressed at equal levels in the reaction as determined by autoradiography (Fig. 21C). They were then tested for interaction with RAC3 by GST-pulldown assay using a GST fusion of the RAC3 receptor-interacting domain (RID) in the presence of 1 μM all-trans retinoic acid (Fig. 21A, left). GST-RID pulled down a significant amount of wild-type ^{35}S -RAR α , compared with only minimal binding to GST alone. However, mutation of any of the cofactor pocket residues drastically reduced this interaction. RAR α V240R, L261R, or E412K, from helices 3, 5, and 12 respectively, each abolished RID-receptor binding such that only a minimal background signal remained. RAR α F249R from helix 4 retained some interaction with GST-RID, but at a very low level compared to wild-type receptor. Equal amounts of GST-RID were used in each reaction, as determined by Coomassie Blue staining (data not shown). Therefore, these results clearly indicate that specific, conserved residues within the predicted RAR α cofactor-binding pocket and an intact charge clamp are required for receptor interaction with the RAC3-RID.

Since it is unclear how RXR, the common heterodimeric partner of non-steroid nuclear receptors, interacts with coactivators, we next analyzed the binding of RXR α to the RAC3-RID and characterized the RXR α residues required for this interaction. Here, double mutants were made in the proposed cofactor-binding pocket of full-length RXR α (Fig. 20). The mutations were: L276A/V280A from helix 3 (m1) and V298A/L301A from helix 5 (m2). Wild-type and mutant receptors were then labeled with ^{35}S -

methionine and tested for interaction with the RAC3-RID by GST-pulldown assay in the presence of 9-cis retinoic acid (Fig. 21B). Similarly to RAR α , either mutant receptor was impeded in its interaction with the RAC3-RID, with RXR α L276A/V280A abolishing the interaction and RXR α V298A/L301A retaining some minimal, but detectable binding.

Intriguingly, several recent studies have determined that the nuclear receptor corepressors SMRT and NCoR contain an LXXLL-like motif, termed the CoRNR box, that is required for interaction with unliganded TR and RAR (34, 73-75). This domain consists of the consensus sequence LXXI/HIXXXI/L and also forms an α -helix. The motif also appears to bind to the same hydrophobic pocket in the receptor as coactivators, but may not require the charge clamp formed by residues from helices 3 and 12, for the CoRNR box is longer than the LXXLL motif and would not fit in the clamped pocket conformation (73). Therefore, we wished to determine if the same residues within the RAR α hydrophobic groove that were required for RAC3 binding were also critical to the binding of the corepressor SMRT. A similar GST-pulldown assay using a GST fusion of SMRT 982-1291, which has previously been demonstrated to be sufficient for binding to RAR α , was used to assess the effects of the RAR α mutations on corepressor binding in the absence of ligand (Fig. 21A, right) (103). GST-SMRT 982-1291 pulled down significant amounts of wild-type RAR α compared to GST alone. The V240R, F249R, and L261R mutations each inhibited the interaction, with F249R and L261R more or less abolishing the binding to the receptor, while V240R had a slightly more modest effect. As expected, mutation of E412 in helix 12 did not alter the SMRT-RAR α interaction, consistent with observations that helix 12 is not required and actually inhibits corepressor

interactions with nuclear receptors (23). This also suggests that an intact charge clamp is not required for corepressor binding. Similar results were obtained with NCoR (data not shown). Therefore, the above data demonstrate that RAR α uses a similar surface for interaction with coactivators and corepressors with the exception of helix 12, which is required for binding of coactivators, but not corepressors. However, since mutation of helix 4 retains some interaction with RAC3 while mutation of helix 3 retains some SMRT binding, it also appears that the components of the cofactor binding pocket do make slightly different contributions to binding different classes of cofactors.

B. RAC3 recruitment to DNA-bound Retinoid Receptors

We have demonstrated above that RAC3 NR boxes ii and iii are critical for interaction with the VDR-RXR heterodimer bound to a DR3 response element while NR boxes i and ii control the interaction with RAR-RXR bound to a DR5 element. Furthermore, we found that helix 12 of RXR inhibits RAC3 binding to VDR-RXR or RAR-RXR, for deletion of this domain strongly enhances the shift of these heterodimers by the RAC3-RID. We wished to extend these studies in order to investigate the relative contributions of the respective cofactor binding pockets of RAR α and RXR α to RAC3 interaction with DNA-bound RAR α -RXR α . To do this, we first performed electromobility shift assays with the mutant RARs heterodimerized with wild-type RXR α and tested their abilities to recruit RAC3 to the ³²P-DR5 in the presence of retinoic acid (Fig. 22). Again, we observed a significant shift of wild-type RAR α -RXR α by the

RAC3-RID on a DR5 element (Fig. 22, lane 2). However, mutation of each of the helices making up the cofactor binding pocket of RAR α more or less completely inhibited this interaction, without significantly affecting heterodimer formation (lanes 3-10). To confirm these observations, this experiment was repeated using the helix 12-truncated RXR α -443, for deletion of RXR α helix 12 enhances coactivator binding to the heterodimer (Fig. 23, lane 3). Indeed, mutation of helices 3, 5, or 12 abolishes recruitment of the RAC3-RID while mutation of helix 5 (F249R) significantly reduces this binding (Fig. 23, lanes 5, 7, 9, 11). To extend this analysis to corepressor recruitment by retinoid receptors, similar gel-shift assays were performed using a purified GST-SMRT 982-1495 fusion protein in the absence of hormone (Fig. 24). This SMRT fragment shifted a portion of the receptor complex to a slower migrating form, while the entire complex was shifted in the presence of RXR α -443 (Fig. 24, lanes 2, 4). Therefore, the AF-2 domain of RXR can also inhibit corepressor binding to DNA-bound nuclear receptors in the absence of hormone, similar to its effects on coactivator binding in the presence of hormone (Fig. 10). Again consistent with GST-pulldown data (Fig. 21), mutation of helices 3, 4, or 5 strongly inhibited SMRT interaction, with the V240R mutant still binding weakly (Fig. 24, lanes 6, 8, 10). Mutation of helix 12 (E412K) expectedly had no effect on recruitment of SMRT (lane 12) compared to wild-type RAR α . Finally, the same result was obtained when this experiment was repeated with RXR α -443, except that the residual binding of SMRT to RAR α V240R/RXR α -443 was more evident (Fig. 25). Therefore, these studies support the hypothesis that RAR α uses an overlapping, but not identical surface to bind coactivators and corepressors. They also

reveal that the AF-2 domain of RXR α is antagonistic to SMRT recruitment to RAR α /RXR α in the absence of hormone, similar to its effects on RAC3 recruitment in the presence of hormone.

Although RXR α was able to bind the RAC3-RID in GST-pulldown assays (Fig. 21), it is not currently known if RXR α makes contacts with coactivators in the context of DNA-bound heterodimeric nuclear receptor complexes. To address this question, the cofactor pocket mutants of RXR α were used in gel-shift assays with wild-type RAR α and the RAC3-RID (Fig. 26). As a positive control, the RID significantly shifted the wild-type heterodimer (lane 2) while inclusion of RAR α V240R abolished recruitment of coactivator completely (lane 4). In contrast, the RXR α mutations had a more modest effect on coactivator binding. RXR α m1 or m2 decreased the interaction, but a detectable shift by the RAC3-RID was still observed (lane 6,8). As expected, the heterodimer in which both receptors were mutated failed to bind the RAC3-RID (lane 10). In order to confirm these observations, we repeated the experiment using RXR α -443 instead of wild-type RXR α (Fig. 27). As demonstrated previously, the RAC3-RID strongly shifts the RAR α /RXR α -443 heterodimer compared to the wild-type heterodimer (lanes 2 and 4). When RXR α -443 harboring the V298A/L301A mutation is dimerized with wild-type RAR α , this shift is reduced somewhat but a strong interaction with the RAC3-RID is still evident (lane 6). Therefore, it can be concluded that the RAR α cofactor binding pocket is required for the recruitment of coactivator while the RXR α pocket contributes, but is not essential to this interaction. It is likely that the RAR

portion of the heterodimer serves as the primary docking point for coactivator and that any interaction between RXR α and coactivator is secondary and not essential.

As demonstrated above, the contribution of RXR α to coactivator binding by the RAR α -RXR α heterodimer appeared minimal. However, mutation of the RXR α cofactor binding pocket did severely diminish the interaction with the RAC3-RID in solution, as evident from the GST-pulldown assay (Fig. 21). In order to investigate these differences further, we analyzed the interactions between the RAC3-RID and a DNA-bound RXR α using a 32 P-labeled DR1 element, which has been demonstrated to support binding by RXR α -RXR α homodimer. In vitro translated wild-type RXR α bound the DR1 probe in the presence of 9-cis retinoic acid and the homodimer was shifted to a slower migrating form upon addition of the RAC3-RID (Fig. 28, lanes 1,2). In contrast, when the RXR α L276A/V280A or V298A/L301A mutants were used in the binding reaction, they were unable to bind to the RID (lanes 4, 6). However, the L276A/V280A mutant homodimer displayed considerably weaker DNA binding, thus this mutation may affect homodimerization or the overall structure of the protein to a greater extent than the V298A/L301A mutation, which bound DNA as well as wild-type RXR α . These results indicate that the RXR α cofactor binding pocket is critical to coactivator binding to the RXR homodimer, but plays a minimal role in coactivator binding to a RAR α -RXR α heterodimer.

C. Effect of RAR α mutations on transcriptional activity in vivo

In order to understand the functional consequences of mutating the cofactor binding site of RAR α in vivo, we performed transient transfection assays to investigate the transcriptional activity of these mutants compared to wild-type RAR α (Fig. 29). As a control, an additional mutant RAR α , RAR α -403, was also tested for transcriptional activity. RAR α -403, in which helix 12 has been deleted entirely, has previously been demonstrated to constitutively repress transcription due to enhanced corepressor binding and failure to recruit coactivator upon hormone binding (23). HEK293 cells were co-transfected with the expression plasmid for each receptor along with a luciferase reporter containing the RAR response element from the RAR β promoter and a beta-galactosidase plasmid that allows normalization of luciferase activity. Transfected cells were also treated with 50 nM all-trans retinoic acid or solvent only for 24 hours before harvesting. In the absence of hormone, RAR α had little activity compared to the empty vector alone, while RAR α -403 repressed transcription below the basal level as expected. In addition, the RAR α V240R, F249R, and L261R mutants all had no effect on reporter expression. However, the helix 12 point mutant, RAR α E412K, did decrease basal expression somewhat, although not to the extent of the helix 12 truncation, RAR α -403, consistent with the ability of RAR α E412K to retain corepressor binding in vitro.

In the presence of hormone, the reporter alone was stimulated 2-fold, likely due to the activation of endogenous retinoid receptors. Transfection of wild-type RAR α resulted in an additional 4-fold enhancement in reporter expression. As expected, RAR α -403 retained the ability to repress transcription even in the presence of ligand

while each of the point mutations strikingly inhibited transcriptional activation by RAR α . RAR α F249R retained the most activity, consistent with its ability to still bind the RAC3-RID weakly in vitro. However, RAR α V240R, L261R, and E412K actually displayed a slight dominant negative effect on the RARE-driven reporter. This finding can likely be attributed to the formation of inactive heterodimers with endogenous RXR, thereby titrating away the formation of active, endogenous RAR-RXR heterodimers in 293 cells. Therefore, these observations correlate with the above in vitro data in implicating specific residues in the cofactor binding pocket of RAR α as being critical to both the interaction with RAC3 and transcriptional activation upon hormone binding. It also suggests that recruitment of coactivators such as RAC3 is required for transcriptional activation by RAR α in vivo.

In order to more clearly investigate the role of the RAR α cofactor binding pocket in transcriptional repression in vivo, the V240R, F249R, and L261R point mutants were subcloned into the RAR α -403 expression plasmid. Since RAR α -403 represses transcription more strongly than the full-length receptor, the effects of these mutations on this repression activity should be more evident in the context of the truncated RAR α . Co-transfection of RAR α -403 with the RARE-driven reporter resulted in a significant repression of basal activity in the absence of hormone (Fig. 30). However, expression of either RAR α -403 V240R, F249R, or L261R each abolished the ability of RAR α -403 to repress transcription, consistent with the reduction in interaction with the corepressor SMRT in vitro. This suggests that recruitment of nuclear receptor corepressors such as

SMRT is required for RAR α to repress transcription in vivo, for mutations that abolish corepressor interaction also abolish repressor activity.

D. Effects of RXR α mutations on transcriptional activity in vivo

We next performed experiments to characterize transcriptional activation by RXR α in vivo by co-transfecting 293 cells with the wild-type receptor or cofactor pocket mutants with a luciferase reporter driven by a DR1-containing promoter (Fig. 31A). Treatment of cells transfected with empty vector with 9-cis retinoic acid had little effect on reporter activity while transfection of wild-type RXR α strongly stimulated expression approximately 22-fold. RXR α L276A/V280A and V298A/L301A were much less efficient in transcriptional activation, consistent with their impaired abilities in binding coactivator in vitro. However, in contrast to the mutated RAR α , the mutated RXR α was still able to activate transcription to some extent. Several possibilities may explain this observation. First, RXR α V298A/L301A retained some RAC3 binding in vitro, albeit very weak. Secondly, RAC3-independent mechanisms of transcriptional activation may also contribute to RXR α activity. This hypothesis contrasts with RAR α , in which coactivator binding appeared to be required for activation. It is also possible that hormone treatment is activating endogenous retinoid receptors at the DR1-driven reporter.

A much different result was obtained when a similar experiment was performed to analyze the RAR α /RXR α heterodimer. RAR α was cotransfected with wild-type or

mutant RXR α and a reporter under the control of a DR5 response element (Fig. 31B).

All-trans retinoic acid, which is specific for RAR α , stimulated this reporter without overexpression of receptors, likely by activating endogenous retinoid receptors.

Overexpression of wild-type RAR α /RXR α further enhanced reporter expression nearly 5-fold. Interestingly, wild-type RAR α coexpressed with RXR α V298A/L301A also did not alter receptor activation of the DR5 reporter, in contrast to its function at the DR1-driven reporter, where it was 4-fold less active than the wild-type receptor. These findings are consistent with our gel-shift data, which demonstrated that the RXR α cofactor binding pocket was critical to RAC3 interaction with the RXR α -RXR α homodimer at a DR1 element, but only made minor contributions to coactivator binding to the RAR α -RXR α heterodimer at a DR5 element. However, the RXR α L276A/V280A mutation did slightly reduce transcriptional activation. This may be due to the more serious effects on overall RXR α structure that occur upon mutating these residues, as described above. It is also possible that helix 3 may be more important to RXR α activity relative to helix 5.

In summary, several novel aspects have been uncovered concerning the structural requirements of retinoid receptors in the recruitment of coactivators and corepressors.

First, the binding pocket of RAR α for these cofactors does overlap to a significant extent.

Nuances do exist, however, for an intact helix 12 is required for RAC3 interactions in the presence of hormone but not for SMRT interactions in the absence of hormone.

Furthermore, although mutation of helices 3, 4, and 5 does significantly inhibit binding to both cofactors, it appeared that intact helix 4 was slightly less critical to recruitment of

RAC3 while intact helix 3 was less important to the recruitment of SMRT. However, one cannot rule out the possibility that the effects of these mutations were due to more global conformational changes in the receptor's structure rather than the inhibition of interactions with a specific domain within the receptor. Second, recruitment of these cofactors in vitro correlates perfectly with the transcriptional activity of the receptors in vivo, since RAC3 interaction was required for activation while SMRT binding was essential to repression. Third, the coactivator pocket of RXR α contributes differently to RAC3 binding depending on the receptor complex. It is required for coactivator regulation of the RXR α homodimer, but contributes minimally to the RAR α /RXR α heterodimer. Lastly, the AF-2 domain of RXR α is clearly a vital regulatory domain in governing interactions between cofactors and the RAR α /RXR α heterodimer, for as in the case of the liganded heterodimer and RAC3 binding, this region also inhibits SMRT binding to the unliganded heterodimer.

CHAPTER V

CONCLUSIONS AND DISCUSSION

A. Conclusions

In this thesis, I have characterized in detail the mechanism by which the coactivator RAC3 regulates the function of nuclear receptors. The centrally located NR boxes, which are comprised of α -helical LXXLL motifs, have been demonstrated to be absolutely critical to the interactions between RAC3 and the various receptors, namely the vitamin D, estrogen, and retinoid receptors. Furthermore, these receptors also had a preference for specific RAC3 NR boxes. It was discovered that NR box iii is required for VDR binding, while NR box ii is essential for RAR α binding, and NR boxes i, ii, and iii are involved, but not individually required for ER β interaction. Consequently, the specific motifs were also crucial to the ability of RAC3 to enhance transcriptional activation by these receptors in vivo. The C-terminal NR boxes of RAC3 were also analyzed in terms of the transcriptional activation function of the coactivator. A single NR box in the RAC3-AD proved to be necessary for activation by RAC3. Mutation of this motif not only abolished the activity of RAC3, but also severely impaired its interaction with the general transcriptional coactivator CBP. However, multiple mechanisms of activation by RAC3 were uncovered, for this mutation slightly reduced RAC3 coactivation of RAR α , but not ER β . In terms of the receptors themselves, it was discovered that the coactivator and corepressor binding pockets overlap to a large extent,

with subtle structural differences being evident, however. Recruitment of these cofactors was also found to be essential to the transcriptional activity of the receptor *in vivo*. Finally, it was demonstrated that the AF-2 domain of RXR α was a key regulatory module governing the interaction of both RAC3 and SMRT to retinoid receptors.

B. RAC3 NR boxes Regulate Interactions with Nuclear Receptors and CBP

The NR boxes are highly conserved among the SRC family of coactivators (104). Our data and that of others clearly reveal that multiple motifs are necessary for high affinity interactions with nuclear receptors (64, 67, 70, 71). Furthermore, this work has uncovered receptor-specific preferences for the different NR boxes of the RAC3 receptor-interacting domain. NR box iii was required for VDR binding, while NR box ii was essential for RAR α binding, and NR boxes i, ii, and iii were involved, but not individually required for ER β interaction. Peptides corresponding to these motifs were able to compete with the RAC3-RID for receptor binding. The integrity of the motifs themselves was also important, for mutations in specific NR boxes inhibited RAC3 interactions with these receptors *in vitro*. With the DNA-bound VDR/RXR α heterodimer, we found that mutation of NR boxes ii or iii of RAC3 weakens the interaction with the RAC3-RID. In contrast, NR box iii of RAC3 is the only critical motif for interaction with VDR in solution. Therefore, it is likely that each motif binds to each monomer of the receptor heterodimer, consistent with the structure of a PPAR γ -LBD dimer co-crystallized with a fragment of SRC1 containing two NR boxes (34). Biochemical data

supporting this hypothesis is evident in a study of TRAP220 interactions with nuclear receptors (105). TRAP220 links certain receptors to the TRAP complex, a large group of polypeptides initially demonstrated to be required for the activity of the thyroid hormone receptor (106). TRAP 220 contains two consensus NR boxes, with RXR preferring box 1 and its partner (VDR, TR, or PPAR) having a clear preference for NR box 2, as shown by in vitro binding assays. Both motifs are required for efficient interaction of TRAP220 with DNA-bound TR/RXR or VDR/RXR and optimal coactivation of VDR-mediated transcriptional activation. Similar results were reported with the TRAP220 homolog of the DRIP complex, DRIP205 (107). Mutation of NR box i of RAC3 also drastically reduced interaction with RXR α , while having much weaker effects on RAC3 interactions with VDR or RAR α . Therefore, for RXR α heterodimers, motif i of RAC3 likely contacts RXR α and motifs ii and iii are utilized by the partnering monomer. The above data on ER β suggest that all three NR boxes of the RAC3-RID are involved for a wild-type interaction while the presence of two motifs is sufficient for a strong interaction, motif ii being most important. In light of this finding, RAC3 may utilize motif ii in combination with motif i or iii for efficient interaction with the ER β homodimer. The integrity of the other motif may be critical to the overall conformation of the coactivator, or potentially make an additional contact with another region of the receptor. Support for the latter possibility can be found in recent studies detailing the enhancement of the N-terminal AF-1 activation function of nuclear receptors by SRC coactivators (38, 41). The presence of multiple NR boxes also likely provides coactivators the flexibility to interact with a broad range of nuclear receptors, resulting in the different preferences that are

observed between nuclear receptors and distinct motifs, depending on the precise structural nuances of each receptor-coactivator interface. This is evident upon comparing the NR box requirements of the VDR/RXR α heterodimer versus those of VDR/RXR α -443. Deletion of the AF-2 helix of RXR α not only enhances RAC3-RID binding to the heterodimer, but also switches the NR box preferences from motifs ii/iii to motifs i/iii. Finally, amino acids flanking the NR boxes also likely contribute to the specificity of interaction (71, 108), for, despite the high homology between the RAC3 NR boxes, peptides comprising each motif and surrounding residues displayed different affinities for VDR or ER β binding. Thus, it is clear that the multiple NR boxes do not serve merely redundant functions.

Importantly, this receptor-specific code has also been analyzed *in vivo* in terms of transcriptional coactivation of nuclear receptors by RAC3 via transient transfection assays. Consistent with the *in vitro* interaction data, mutation of NR box ii or iii blocked the ability of RAC3 to enhance the activity of VDR in CV-1 cells. In contrast, mutation of NR boxes i, ii, or iii reduced, but did not abolish RAC3 coactivation of ER β . Similar studies have been performed with SRC-1 using site-directed mutagenesis and antibody microinjection assays (71). The requirement of specific NR boxes for transactivation of reporter genes by different receptors was determined by injecting anti-SRC-1 antibodies into cells along with rescuing plasmids for wild-type or NR box mutants of SRC-1. Anti-SRC-1 IgG completely abolishes transcriptional activation by ER, PR, RAR, TR, and PPAR γ , while coinjection of wild-type SRC-1 rescues receptor function. Mutation of NR box ii prevented rescue of ER function in SRC-1 immunodepleted cells while NR boxes

ii and iii were required for rescue of RAR and TR activity and boxes i and ii for PR activity. Furthermore, in the case of PPAR γ , different ligands elicited different NR box requirements for SRC-1 coactivation. Troglitazone-bound PPAR γ preferred NR box ii over box i while the opposite was observed in indomethacin treated cells. Together, these data support receptor-specific LXXLL motif requirements for coactivation function and receptor interactions that account for the presence of multiple NR boxes within SRC coactivators.

A role also has been revealed for the C-terminal NR boxes of RAC3 in the transcriptional activation function of the coactivator, for RAC3 can activate transcription when fused to the Gal4 DNA-binding domain. NR box v of RAC3 proved to be required for wild-type trans-activation by RAC3. Mutation of this motif not only abolished the activity of RAC3, but also nearly abolished the ability of RAC3 to interact with the general transcriptional coactivator CBP. This observation suggests that one mechanism by which RAC3 is able to activate transcription is via recruitment of CBP. However, other, CBP-independent mechanisms of activation by SRC members clearly exist (41, 43, 64). In support of this, we found that mutation of NR box v had no effect on the ability of RAC3 to coactivate ER β . RAC3 may recruit other factors such as CARM1 or the DRIP/TRAP complex, or target the general transcription machinery in order to enhance ER β activity. In contrast, the NR box v mutation did slightly reduce coactivation of RAR α , indicating that CBP recruitment may be contributing factor to RAC3 coactivation of RAR α .

C. Mechanisms of Cofactor Recruitment by Retinoid Receptors

The residues required for cofactor interaction and the contribution of these amino acids to the transcriptional activity of the receptor were also investigated. Recent crystallographic evidence has detailed the formation of a hydrophobic pocket, induced by ligand binding to nuclear receptors, which serves as the docking surface for the LXXLL motif of coactivators (31-34). This pocket consists of helices 3, 4, 5, and 12 of the ligand-binding domain and also includes the charge clamp, a conserved glutamate from helix 12 and lysine from helix 3, which positions the motif within the pocket. Based on the corresponding residues interaction with the LXXLL motif in the crystal structures of hER α , hTR β , or hPPAR γ complexed with SRC fragments, a highly conserved amino acid from each of these helices of human RAR α was selected for analysis. We found that mutation of valine 240, phenylalanine 249, or lysine 261, from helices 3, 4, and 5, respectively, each strikingly inhibited RAR α interactions with RAC3 in vitro and transcriptional activation by the receptor in vivo. Mutation of the charge clamp glutamate, E412, had the same effect. We concluded from these findings that coactivator interaction is required for transcriptional activation function of RAR α . This also suggests that any contribution of the RAR α AF-1 domain to the overall activity of the receptor is minimal. Interestingly, the V240, F249, or L261 mutations also impaired the interaction of RAR α with the nuclear receptor corepressor SMRT. In the context of RAR α 403, a helix 12 truncated receptor that constitutively represses transcription, these mutations also blocked this repressor activity. Thus, corepressor recruitment is required

for transcriptional repression by RAR α . This data is consistent with recent studies that describe an LXXLL-like motif, termed the CoRNR box, which is required for corepressor interaction with unliganded nuclear receptors and may also bind to the same hydrophobic pocket on the receptor as coactivators (73-75). However, unlike coactivators, the charge clamp and helix 12 are likely not involved in the binding of corepressors, based on the length of the CoRNR box. Our finding that mutation of glutamate 412 from helix 12 has no effect on the RAR α -SMRT interaction support this hypothesis.

Finally, we have investigated the ability of RXR α , the common heterodimeric partner for non-steroid nuclear receptors, to interact with RAC3. In solution, RXR α was able to bind the RAC3-RID, and mutation of specific cofactor-pocket amino acids inhibited this interaction. Intriguingly, however, the contribution of the RXR α cofactor pocket contributed differently to coactivator recruitment to DNA-bound nuclear receptors depending on the particular dimer examined. In the case of the RXR α /RXR α homodimer bound to a DR1 element, the cofactor pocket was required in order to bind the RAC3-RID. Consistently, mutation of this pocket drastically reduced the ability of RXR α to activate transcription in vivo from a DR1-driven reporter. A different pattern was evident upon examining the RAR α /RXR α heterodimer. Here, the RXR α cofactor pocket only contributed somewhat to coactivator binding at a DR5 element, while the RAR α pocket was absolutely required. In support of this in vitro data, the RXR α mutant had only slight effects on RAR α /RXR α transcriptional activity at a DR5-driven reporter in vivo. In summary, these observations suggest that RAR α is the primary docking point

for coactivator binding to the RAR α /RXR α heterodimer, but that RXR α can also recruit RAC3 to the RXR α /RXR α homodimer. In both cases, an intact cofactor binding pocket is essential to the recruitment of RAC3.

D. The AF-2 domain is a Critical Regulator of Cofactor Recruitment

The finding that the AF-2 domain of RXR α can interfere with RAC3-RID binding to a DNA-bound VDR/RXR α or RAR α /RXR α heterodimer is consistent with studies suggesting allosteric inhibition of coactivator binding to RAR/RXR by the RXR AF-2 domain (100). This inhibition may be the result of competition between the AF-2 domain of RXR and the LXXLL motif for the coactivator binding site on the other receptor. In the antagonist-bound ER α -LBD and RAR/RXR-LBD crystal structures, the AF-2 domain occupies the coactivator binding groove, mimicking the hydrophobic interactions of the NR box peptide with this domain in the agonist-NR box peptide-receptor complex (31, 77). Biochemical studies with RAR/RXR and SRC1 support these observations, for binding of RAR- and RXR-specific ligands enhance SRC1 interaction with the receptor dimer relative to the interaction in the presence of either ligand alone (100). Presumably, one ligand binding recruits a single NR box to the receptor dimer, which displaces the AF-2 domain from the coactivator binding site and relieves allosteric inhibition, allowing the second ligand to bind the other receptor monomer. This, in turn, enhances the interaction with coactivator by recruiting a second NR box (100). In the case of wild-type receptors, hormone does stimulate RAC3-RID binding to the heterodimer, but only

weakly compared to the VDR/RXR α -443 or RAR α /RXR α -443 dimers, where a very strong, ligand-dependent interaction is observed. These observations are confirmed by in vivo studies demonstrating that RAC3 can coactivate VDR/RXR α -443 activity to a greater extent than VDR/RXR α activity. Hormone binding and RID recruitment must not be able to displace every RXR α AF-2 domain from the coactivator binding site of the partnering receptor, thus fewer RID molecules are able to bind in the presence of the RXR α AF-2 domain. This suggests that the AF-2 domain of RXR α plays a critical role in regulating RAC3 modulation of receptor function. However, other possibilities may explain this finding, foremost being the hypothesis that deletion of the AF-2 domain of RXR α results in a conformational change of the VDR/RXR α -443 dimer that enhances its affinity for the RAC3-RID. Our data demonstrating that the coactivator and corepressor binding sites of RAR α overlap to a large extent further support the critical role of the AF-2 domain in regulating cofactor interactions with nuclear receptors. Intact helices 3, 4, and 5 are required for both RAC3 binding to liganded RAR α and SMRT binding to unliganded RAR α . However, helix 12, which comprises the AF-2 domain is only required for coactivator binding. Therefore, these studies suggest that coactivators, corepressors, and the RXR AF-2 domain all can interact with a similar surface on the partnering monomer. Corepressor may displace the AF-2 domain of RXR and bind to the DNA-bound heterodimer in the absence of hormone. Thus, helix 12 is not required for corepressor-receptor interactions. Our data demonstrating that mutation of the highly conserved glutamate in RAR α helix 12 has no effect on the RAR α -SMRT interaction support this hypothesis. Subsequent ligand binding by the receptor induces a

conformational change in helix 12 that may displace corepressor, thereby facilitating coactivator binding. This indicates that helix 12 is essential for coactivator binding. However, it does appear evident from the data presented here that slight structural differences do exist between the coactivator and corepressor pockets in addition to the contribution of helix 12. Specifically, RAC3 retained some residual binding to RAR α F249R while RAR α V240R had somewhat weaker effect on interaction with SMRT. Therefore, it is possible that helix 4 may be less important to coactivator binding while helix 3 is not as critical to the binding of corepressors.

E. Functional Differences between Members of the SRC Family

Our data demonstrates for the first time that RAC3 can enhance the transcriptional activation function of the VDR and ER β , and that this coactivation activity depends on different NR box requirements. Several other cofactors have been found to stimulate VDR activity, including SRC1, GRIP1/TIF2, NCoA-62, and the DRIP complex (109-112) while SRC1 can coactivate the ER β (113). The role of multiple coactivators in the function of nuclear receptors in vivo is one of the most important remaining questions to be answered concerning the function of the SRC family. Although all three members do possess similar properties in terms of interactions with nuclear receptors and enhancement of transcriptional activation, several reports suggest that their activities are not completely overlapping and particularly outline a division between SRC-1 and TIF2/GRIP1 versus RAC3/ACTR/pCIP/AIB1 functions. For example, microinjection of

expression plasmids for SRC-1 or NCoA-2, but not pCIP, were able to rescue RAR-dependent activation in SRC-1 immunodepleted cells (53). Also, the relative contribution of each coactivator may depend on cell or tissue type and/or coactivator levels in these cells. RAC3/ACTR/AIB-1 is expressed at high levels in placenta, heart, and HeLa cells relative to TIF2 and SRC-1, thus it may serve a more prominent role in nuclear receptor function in these cells (57). In addition, AIB-1 was cloned as a gene that is amplified in ER-positive BT-474, MCF-7, and ZR75 breast cancer cell lines (55). AIB-1 mRNA and protein levels are expectedly higher in these cells as well. SRC-1 and TIF2/GRIP1 are expressed at relatively low levels in these cell lines, suggesting that AIB-1 is specifically involved in the pathogenesis of these tumors. Our data demonstrating that RAC3 can coactivate both RAR α and ER β while TIF2 coactivates only ER β and SRC-1 does not coactivate either receptor support the hypothesis of distinct roles in receptor regulation by the three members of the SRC family of coactivators. Consistently, RAC3 and TIF2 NR box peptides have been demonstrated to have a higher affinity than those of SRC-1 for ER β (114).

However, perhaps the most conclusive evidence for unique functions of SRC coactivators can be found in studies from the O'Malley laboratory detailing SRC-1 and RAC3 knockout mice (60, 61). In the SRC-1 $-/-$ animal, the mice had no external phenotype compared with the wild-type control mice (61). Both males and females displayed normal growth rates and were fertile. However, upon analysis of steroid hormone responses in reproductive tissues, female mice did show decreased estrogen-induced uterine growth while males exhibited decreased testosterone-induced prostate

growth. Females were also inhibited in terms of mammary ductal elongation and alveolar development. An additional study demonstrated that the SRC-1 null mice also displayed a resistance to thyroid hormone (115). These findings suggest that SRC-1 is required for maximal response to hormone in vivo. Interestingly, the coactivator TIF2 was overexpressed by two-fold in these animals while the expression level of RAC3 was unaffected, indicating that TIF2 may compensate for the loss of SRC-1 and that RAC3 may serve a different functional role in steroid receptor activity. This was confirmed in the report of the RAC3 knockout mouse (60). These mice displayed a much more severe phenotype. Males and females had reduced growth rates, likely due to decreased levels of insulin-like growth factor (IGF-1). Furthermore, females were delayed in the onset of puberty and had abnormal reproductive function, as measured by decreased ovulation, decreased pregnancy rates, and decreased litter sizes. Finally, as in the SRC-1 knockout, female RAC3 $-/-$ mice also were impaired in the development of the mammary gland. These two studies clearly outline a distinct biological role for RAC3 versus SRC-1 and TIF2, with RAC3 likely being more critical to overall growth and sexual maturation.

F. SRC-associated Coactivators

In addition to the SRC family of coactivators, many other cofactors have been identified which stimulate the activity of nuclear receptors (116). For the sake of brevity, only those associated with SRC family members will be discussed here.

SRA

Recently, in a search for nuclear receptor cofactors, a novel steroid receptor RNA activator (SRA), was isolated in a yeast-two-hybrid screen using the AF-1 domain of the PR as the bait (117). This coactivator is selective for the N-terminal AF-1 activation domain of nuclear receptors and can reverse estrogen-induced squelching of PR-driven gene expression. Interestingly, SRA is also selective for steroid receptors versus RXR heterodimers, for it enhances only PR, GR, AR, and ER activities while having no effect on the activities of the TR β , RAR γ , RXR γ , or PPAR γ . However, maybe the most surprising characteristic of SRA is that it apparently functions as an RNA transcript, which is evident from several observations. First, efforts to generate SRA-encoded protein *in vitro* or *in vivo* were not successful. Second, several mutant constructs of SRA that disrupt translational start sites or open reading frames were still able to potentiate PR activity. Third, SRA retained coactivation activity in the absence of protein synthesis via cyclohexamide treatment. Finally, SRA transcripts were identified as components of an SRC-1 complex *in vivo* via whole cell fractionation followed by gel filtration chromatography. SRA, detected by RT-PCR, specifically copurified with SRC-1 in the same fractions. SRA mRNA was also efficiently coimmunoprecipitated with SRC-1 antibodies, further supporting the existence of a complex containing SRA and SRC-1. This study suggests that SRA is a novel RNA coactivator that forms a complex with SRC-1 *in vivo* and selectively enhances the activity of steroid hormone receptors via the AF-1 domain.

CARM1

A novel enzymatic activity was attributed to nuclear receptor coactivators with the cloning of CARM1 (Coactivator-associated arginine methyltransferase 1) via a yeast-two-hybrid screen using the C-terminal amino acids 1121-1462 of GRIP1 (80). This region represents a second, CBP-independent activation domain of GRIP1 (41). CARM1 showed extensive homology to the PRMT (protein methyltransferase) family of arginine-specific methyltransferases and interacted with all three members of the SRC coactivator family in vitro. Furthermore, CARM1 contained potent histone methyltransferase activity in vitro, with a preference for histone H3. In vivo, CARM1 enhanced transcription by a Gal4-DBD fusion of GRIP1 1121-1462 and further stimulated GRIP1 coactivation of AR, TR, and ER activities. This coactivation function was dependent upon three amino acids located in the region critical to methyltransferase activity, suggesting that this enzymatic activity is required for CARM1's ability to enhance receptor function. In the absence of GRIP1, CARM1 had no effect on receptor function, thus SRC coactivators are likely required in order to recruit CARM1 to the receptor complex. Overall, the cloning of CARM1 contributes to the mechanism by which SRC coactivators activate transcription through multiple domains. One activation domain may be required in order to recruit CBP/p300 and histone acetylation activity, while the second activation domain recruits CARM1 and histone methylation activity. These multiple enzymatic functions may be promoter specific or cooperate to remodel chromatin and facilitate transcriptional activation.

PGC-1

The cloning and characterization of PGC-1 (PPAR Gamma Coactivator-1) was a critical finding to the field of nuclear receptor coactivators, for it linked coactivator function to the regulation of a specific physiological process, namely adaptive thermogenesis. PGC-1 was isolated in a yeast-two-hybrid screen using PPAR γ 183-505 as the bait and was demonstrated to interact with several members of the nuclear receptor superfamily (118). It also possesses potent coactivation function for PPAR γ and TR activities at the UCP-1 (uncoupling protein-1) promoter, inducing the expression of this mitochondrial protein involved in heat generation in brown fat cells. Consistently, PGC-1 is also upregulated in muscle and brown fat cells upon exposure to cold temperatures. Further studies have demonstrated that PGC-1 enhances mitochondrial biogenesis and oxygen consumption in muscle cells via the induction of UCP-2 and the regulation of NRFs (nuclear respiratory factors), which are transcription factors that regulate genes involved in mitochondrial DNA replication and transcription (119). Finally, a very recent study reports the functional association between PGC-1 and SRC-1 (120). SRC-1, as well as CBP/p300, interacts with PGC-1 in vitro and in vivo and enhances transcriptional activation by a Gal4DBD fusion of PGC-1 in transient transfection assays. These interactions are mediated by SRC-1 782-1139 and p300 1805-2441. Intriguingly, expression of PPAR γ or NRF-1 also enhanced Gal-PGC-1 activity while cotransfection of PPAR γ increased the interaction between PGC-1 and SRC-1 or CBP/p300 in vitro and in vivo. These data support a model of PGC-1 activation in which the interaction with a transcription factor such as PPAR γ stimulates PGC-1 activity by inducing SRC-1

recruitment (120). This recruitment likely is the result of a conformational change in PGC-1 that occurs upon binding to the transcription factor. This study also suggests the SRC coactivator function may be essential to adaptive thermogenesis, for it may be required for transcriptional activation by PGC-1.

G. Coactivator Complexes

Much effort has been made recently to isolate and purify an entire complex of polypeptides that functions to coactivate nuclear receptor function. However, the few studies that have been reported concerning the existence of an SRC coactivator-containing complex are unclear and even contradictory. Xu, et al identified one such complex by incubating T47D cell extracts with purified, histidine-tagged PR immobilized on a Ni affinity column (121). Upon elution, SRC-1 and CBP were found in the PR-containing fraction only in the presence of progesterone. Furthermore, this complex co-immunoprecipitated in the presence of hormone using an anti-CBP antibody and was prevented from assembling upon expression of E1A. However, using gel filtration fractionation of T47D or HeLa cell lysates, McKenna, et al found that SRC-1 and CBP eluted in distinct fractions, implying that these proteins exist in different preformed complexes (122). CBP copurified with the p300/CBP-associated factor PCAF while SRC-1 copurified with TIF2. Therefore, from these studies, it is difficult to get a clear picture of the existence of an SRC complex *in vivo*.

DRIP/TRAP Complex

Several groups also have identified virtually identical complexes that appear distinct from the SRC coactivator complex. Using the VDR-LBD as an affinity matrix, a complex was purified from Namalwa cell extracts termed DRIP (VDR-interacting proteins) that specifically interacts with ligand-bound VDR-LBD (111). The same complex was also purified using affinity chromatography from HeLa cells that constitutively express Flag-tagged TR and named TRAP (TR-associated proteins) (106). Subsequently, this complex was identified as ARC (activator-recruited cofactor) (123) and SMCC (Srb/Mediator coactivator complex) (124). The DRIP/TRAP complex lacks CBP/p300 or SRC proteins (125) and is recruited to the receptor AF-2 domain by the DRIP205/TRAP220 subunit via a single LXXLL motif (125, 126). Additional contacts between the DRIP150 subunit and the AF-1 domain of the glucocorticoid receptor have also been reported, which together with DRIP205, may functionally link the GR AF-1 and AF-2 domains to maximally enhance GR activity (127). Unlike SRC coactivators, the DRIPs/TRAPs have been demonstrated to be required for transcriptional activation by nuclear receptors in cell-free in vitro transcription assays (106, 111). The DRIPs also enhance VDR activity on chromatin-organized templates despite a lack of HAT activity, suggesting a potential unidentified chromatin remodeling function (125). Furthermore, it is evident that this complex plays a more global role in transcriptional activation rather than being specific to nuclear receptors, for ARC was identified as a coactivator for VP16 and p65 (123), while SMCC enhances p53 activity (124).

Swi/Snf complex

The Swi/Snf complex, which contains ATP-dependent chromatin remodeling activity, has also been implicated in nuclear receptor function (128). Initially identified by yeast genetics as a group of gene products which reduced expression of the SUC2 and HO genes involved in sucrose fermentation and mating-type switching, homologs were later cloned from *Drosophila* (Brahma) and mammalian cells (hbrm and BRG-1). The first link to receptor function was revealed by the loss of steroid-induced transcriptional activation by the GR and ER in Swi/Snf mutant yeast strains (129). Additional work on the GR demonstrated that hbrm and BRG-1 could enhance GR activity in mammalian cells and that GR transactivation of chromatin organized templates required the BRG-1 complex (130, 131). This interaction between GR and the Swi/Snf complex was subsequently shown to be mediated by the AF-1 domain of the receptor, suggesting the possibility that the Swi/Snf and SRC complexes may cooperatively enhance receptor function via recruitment to the AF-1 and AF-2 domains, respectively (132). Finally, hbrm and BRG-1 have also been demonstrated to potentiate transcription by the ER and RAR (133).

H. Model of SRC Function

In order to integrate the wealth of data collected on the mechanism of action of SRC coactivators, the following model of SRC function in the regulation of nuclear receptor activity can be proposed (Fig. 32). Hormone binding triggers nuclear

translocation of Type I steroid receptors and the release of the corepressor complex from Type II non-steroid receptors and subsequent recruitment of an SRC coactivator to the target gene promoter. SRC is able to interact with the AF-2 domain of each monomer of the dimer via multiple, α -helical NR boxes located in the receptor-interacting domain. SRC is likely complexed with the RNA coactivator SRA, which enhances AF-1 activity. After initial SRC docking to the receptor, it is able to recruit additional coactivators to the complex. These include CBP/p300, which uses the NR boxes of the SRC transcriptional activation domain for interaction with coactivator, and the CBP/p300-associated factor P/CAF. Additional, direct interactions between CBP/p300 and nuclear receptors and between P/CAF and SRC have also been reported, which may enhance complex formation. Furthermore, SRC is also able to recruit CARM1 to the target gene via a different domain than that required for CBP/p300 binding. Once this complex is assembled, the histone acetylase activities of CBP/p300, P/CAF, and possibly SRC itself, together with the histone methyltransferase activity of CARM1, serve to modify chromatin, thus facilitating the access of additional transcription factors, coactivators such as the DRIP/TRAP complex, and/or the basal transcription machinery to the target gene promoter to activate transcription. Of course, caveats to this model likely exist. For example, the coactivator complex may be comprised of different components depending on the specific nuclear receptor, cell type, or target gene. Different coactivator complex components may create a level of specificity among different receptors that answers the questions surrounding the potential redundancy among the members of the SRC family. Also, with the intense focus on hormone action and plethora of receptor cofactors being

cloned in recent years, it is likely that additional members of the coactivator complex have yet to be identified. In addition, non-histone substrates for the enzymatically active cofactors may be involved, for as described above, CBP/p300 can acetylate non-histone proteins such as ACTR, p53, and TFIIE/TFIIF. Finally, it is possible that the receptor is able to recruit single, pre-formed coactivator complex to the target gene upon hormone binding. However, though the precise details of transcriptional activation by nuclear receptors are still not clear, it is evident that the SRC family of coactivators is critical to receptor function and will continue to warrant investigation into its role in intracellular signaling pathways.

I. Models of Multiple Coactivator Complexes in Nuclear Receptor Function

One of the most important questions remaining to be answered concerning the transcriptional activation function of nuclear receptors is how these coactivators, including the SRC family, CBP/p300, the DRIP/TRAPs, and Swi/Snf, all contribute to receptor function. Several models have been proposed (Fig. 33) (99):

- 1) Sequential: In this model, each coactivator complex is recruited to a given promoter by the receptor in sequence. For example, Swi/Snf may be recruited first in order to remodel the chromatin architecture by nucleosomal disruption/displacement, thereby facilitating the access of subsequent activators. Upon completion of this activity, this

complex leaves the promoter and is replaced by the SRC complex, which contains histone acetylation and methylation activities. The SRC complex may then be dissociated by acetylation of SRC by CBP (94) and exchanged for the DRIP/TRAP complex, which likely targets RNA polymerase and the basal transcription machinery (134). It is unclear whether a given complex actively recruits the next complex or if other signaling events, such as phosphorylation, lead to the exchange of complexes.

- 2) Cooperative: Here, different coactivator complexes may be recruited at the same time to distinct hormone response elements at a given promoter. The SRC and DRIP/TRAP complexes may bind separate receptors at the target gene promoter and together synergize for maximal transcriptional activation. It is possible that the sequences flanking a given response element may contribute to the specificity of coactivator recruitment or that the different complexes can compete for receptor binding.
- 3) Parallel: Finally, it is also possible that different coactivator complexes are recruited to the same target gene in a signal specific manner. For example, signal A, be it a phosphorylation event or the binding of another transcription factor, may result in the recruitment of the SRC complex to a given receptor. On the other hand, signal B may stimulate the recruitment of the DRIP/TRAP complex. This model would allow for different responses to specific signaling pathways that may account for the amazing array of biological functions carried out by nuclear receptors.

CHAPTER VI

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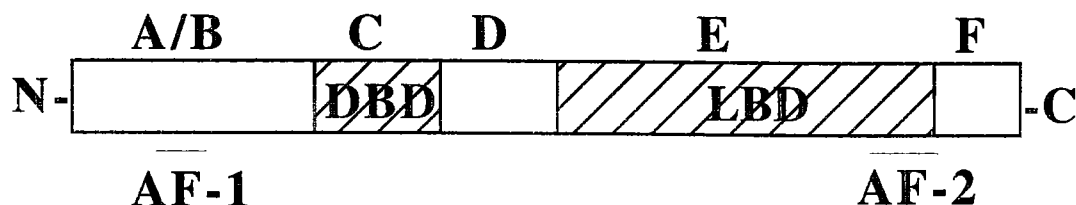
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CHAPTER VII

FIGURES

Nuclear Hormone Receptors



- Ligand-dependent transcription factors
- Bind specific hormone response elements
- Steroid Receptors
 - ER,PR,GR,AR,MR
 - homodimers
- Non-steroid receptors
 - RAR,TR,VDR,PPAR
 - heterodimerize with RXR

Figure 1. Nuclear Receptor Domain Structure. Most members of the steroid/thyroid hormone receptor superfamily share a common domain structure. The N-terminus contains the highly variable A/B domain and the ligand-independent activation function-1 (AF-1) domain. This is followed by the highly conserved DNA-binding domain (DBD). The C-terminus includes the ligand-binding domain (LBD), which also mediates dimerization and cofactor binding. The LBD also contains the ligand-dependent AF-2 transcriptional activation domain.

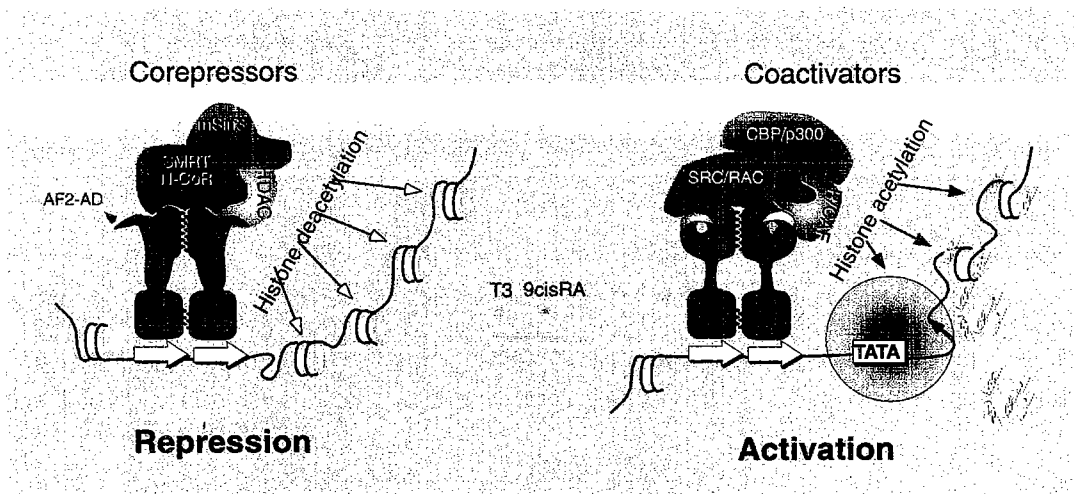
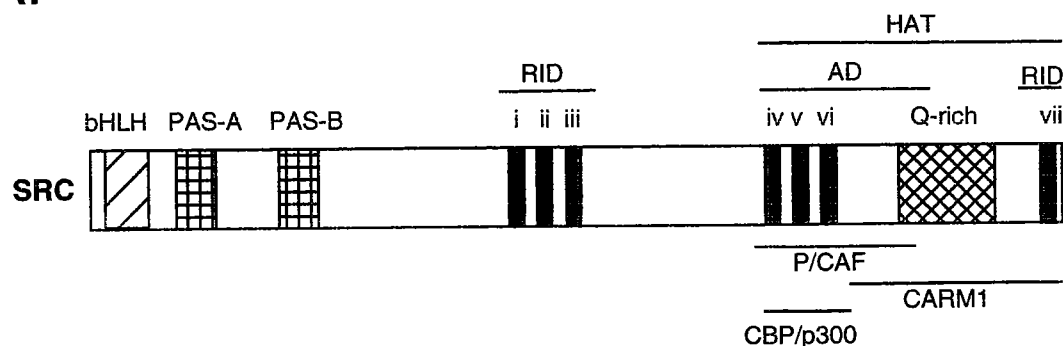


Figure 2. Model of Nuclear Receptor Function. In the absence of hormone, receptors actively repress transcription via interactions with corepressors by mechanisms including histone deacetylation. Ligand binding triggers the release of the corepressor complex and subsequent recruitment of a coactivator complex, which enhances transcriptional activation by the receptor by mechanisms that include histone acetylation.

A.



B.

NR boxes

i.	RAC3	615	SKGHKK	LLQLL	TCSSDD
	TIF2	640	SKGQTK	LLQLL	TTKSDD
	SRC1	632	SQTSHK	LVQLL	TTTAAE
ii.	RAC3	678	LQEKHR	ILHKLL	QNGNSP
	TIF2	683	LKEKHK	ILHRLL	QDSSSP
	SRC1	683	LTERHK	ILHRLL	QEG.SP
iii.	RAC3	730	KKKE..NNA	LLRYLL	DRDDPSD
	TIF2	738	KKKE...NA	LLRYLL	DKDDTKD
	SRC1	739	KKKESKDHO	LLRYLL	DKDE.KD
iv.	RAC3	1025	QNRPLLRNS	LDDLVL	GPPSNLE
	TIF2	1051	QNRQPFSSP	PDDL	CPHPAAE
	SRC1	907	QC...ISSQ	LDELL	CPPTTVE
v.	RAC3	1045	EGQSDERA	LLDQL	HTLL
	TIF2	1071	ESPSDEGA	LLDQL	YLAL
	SRC1	924	EGRNDEKA	LLEQL	VSFL
vi.	RAC3	1069	LEEIDRALG	IPELV	NQ
	TIF2	1093	LEEIDRALG	IPELV	SQ
	SRC1	948	LAELDRAVG	IDKL	V.Q
vii.	SRC1	1424	QTPQAQQKS	LLQQL	TE

Figure 3. SRC family domain structure. (A) Schematic representation of the structural domains of SRC coactivators. RID=receptor-interacting domain, AD=activation domain, HAT=histone acetyltransferase domain. (B) Sequence alignment of the SRC LXXLL motifs.

ER α -LXXLL peptide complex

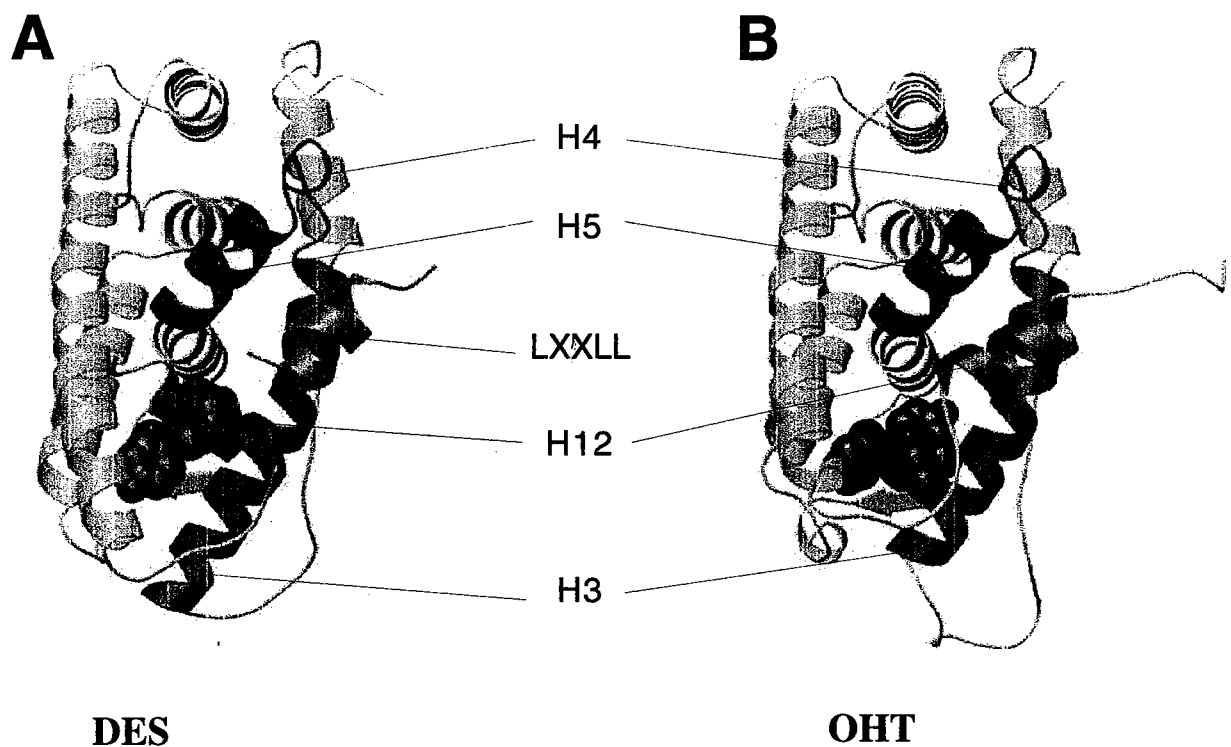


Figure 4. Crystal structure of ER α -LBD complexed with (A) agonist (DES) and GRIP1 NR box ii versus (B) antagonist (OHT). The LXXLL motif is in orange and helix 12 is in purple. In the agonist-bound structure, helices 3, 4, 5, and 12 form a hydrophobic pocket that serves as the docking point for the LXXLL motif of coactivator. However, in the antagonist-bound structure, helix 12 mimics the LXXLL motif, thereby preventing coactivator binding. Adapted from Shiao, et al. Cell 95;927. DES=diethylstilbestrol, OHT=hydroxy - tamoxifen.

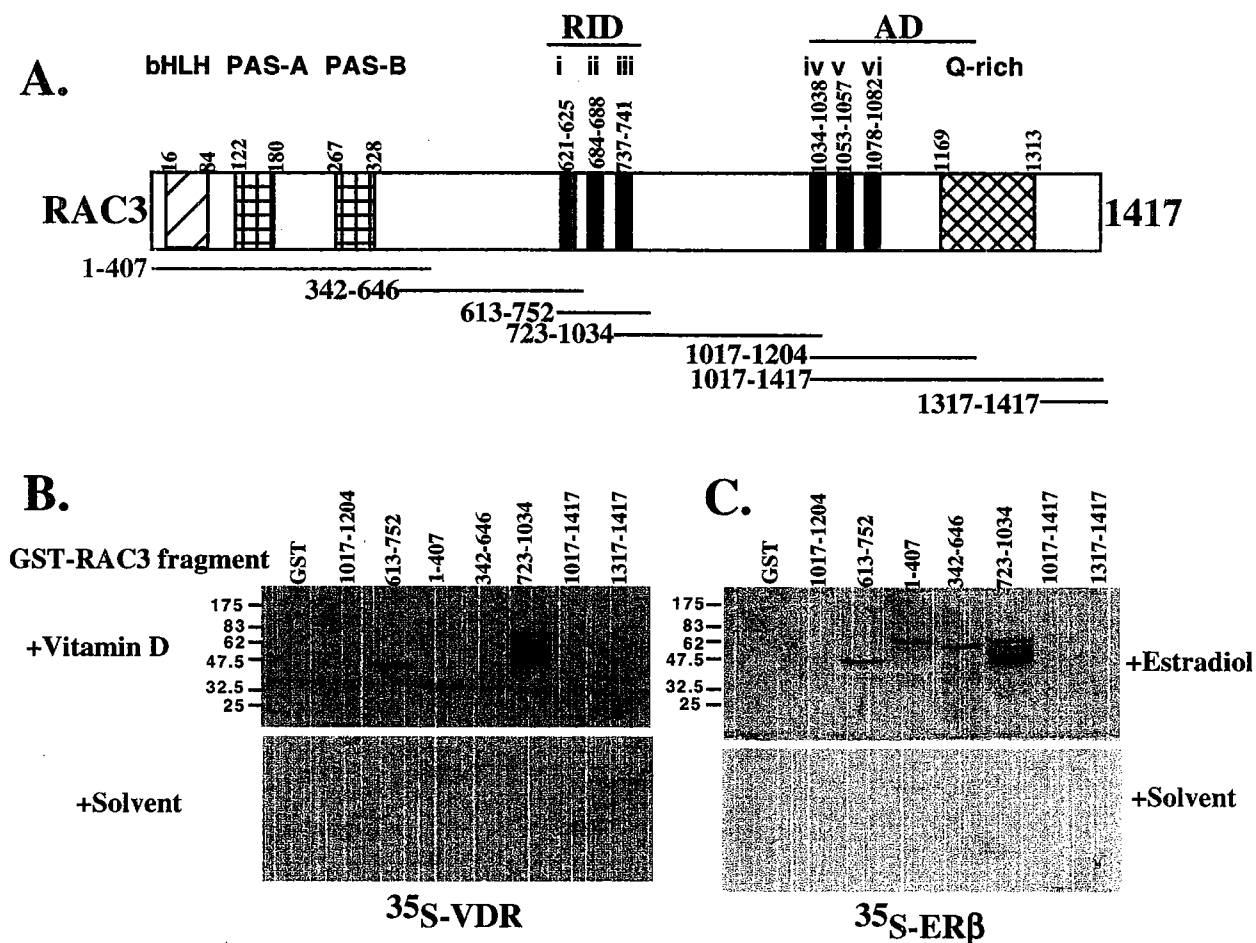


Figure 5. The VDR and ER β interact with different fragments of RAC3. (A) A schematic illustration of RAC3 and its functional domains, as well as the purified GST-RAC3 fragments used for Far-Western assays. RID=receptor-interacting domain, AD=activation domain, i-vi=RAC3 LXXLL NR boxes. (B) Far-western assay using ^{35}S -VDR to probe GST-RAC3 fusion proteins in the presence or absence of $1\mu\text{M}$ 1,25-dihydroxy-vitamin D $_3$. (C) Far-western assay using ^{35}S -ER β to probe GST-RAC3 fusion proteins in the presence or absence of $1\mu\text{M}$ 17 β -estradiol.

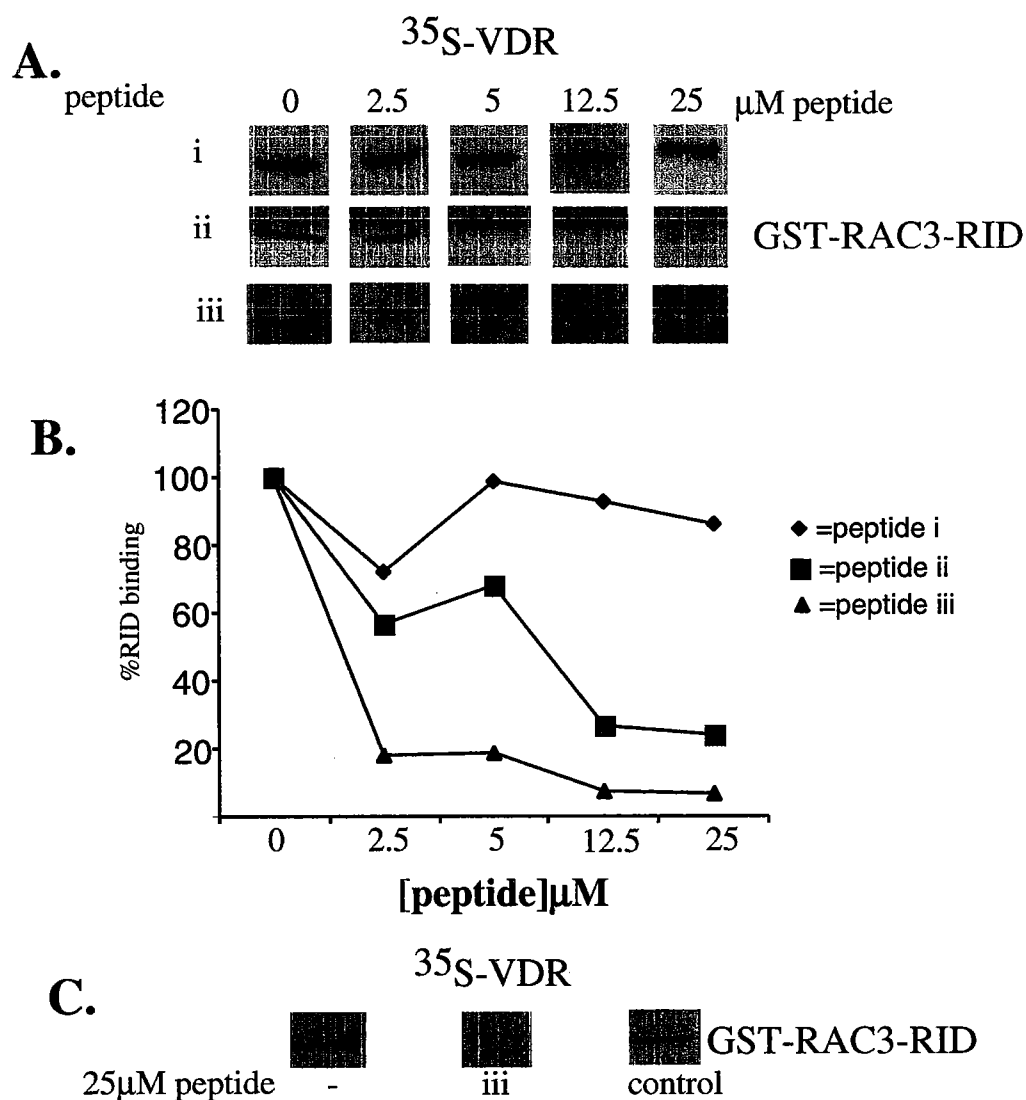


Figure 6. Peptides corresponding to the NR boxes of the RAC3-RID can compete for VDR binding. (A) Far-western assay using ^{35}S -VDR to probe GST-RAC3-RID in the presence of 1 μM vitamin D and given concentration of peptide. (B) PhosphorImager quantitation of the data from (A) plotted as percent GST-RAC3-RID binding to ^{35}S -VDR versus peptide concentration. (C) A control peptide has no effect on the GST-RAC3-RID interaction with VDR.

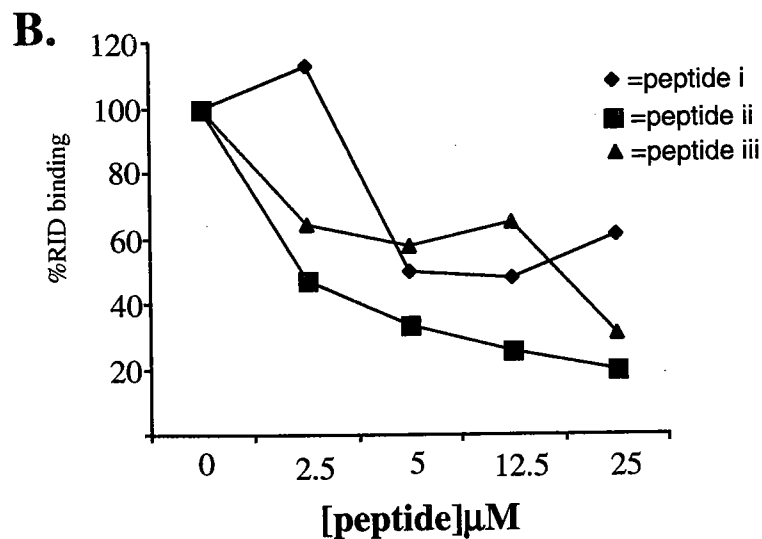
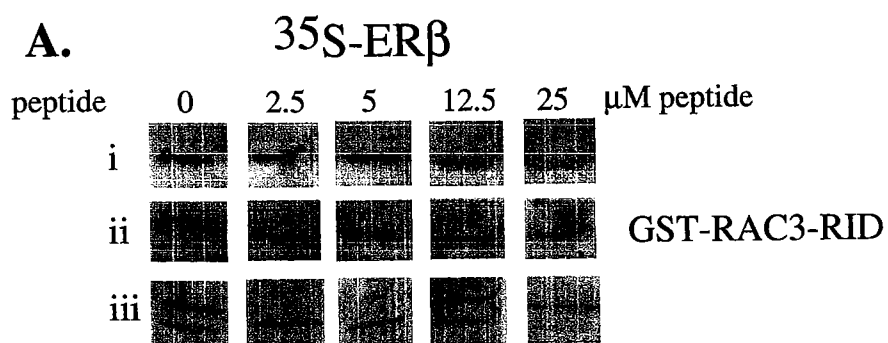


Figure 7. Peptides corresponding to the NR boxes of the RAC3-RID can compete for ER β binding. (A) Far-western assay using ^{35}S -ER β to probe GST-RAC3-RID in the presence of $1\mu\text{M}$ 17β -estradiol and given concentration of peptide. (B) PhosphorImager quantitation of the data from (A) plotted as percent GST-RAC3-RID binding to ^{35}S -ER β versus peptide concentration.

A.

mut i	RAC3 (621-625)	LLQLL → LLQAA
mut ii	RAC3 (685-689)	LHKLL → LHKAA
mut iii	RAC3 (737-741)	LRyll → LRYAA

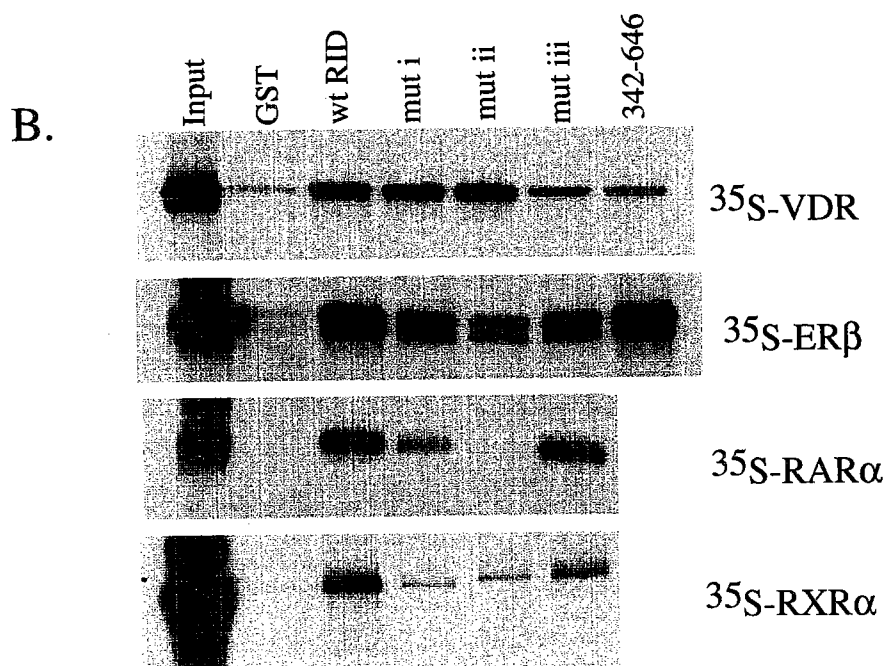


Figure 8. Mutation of RAC3 NR boxes reveal different LXXLL motif preferences for nuclear receptor binding.

(A) Alanine substitution for leucine in each of the RAC3-RID NR boxes.
 (B) GST-pulldown assay of indicated nuclear receptors and GST fusion proteins in the presence of 1 μ M hormone.

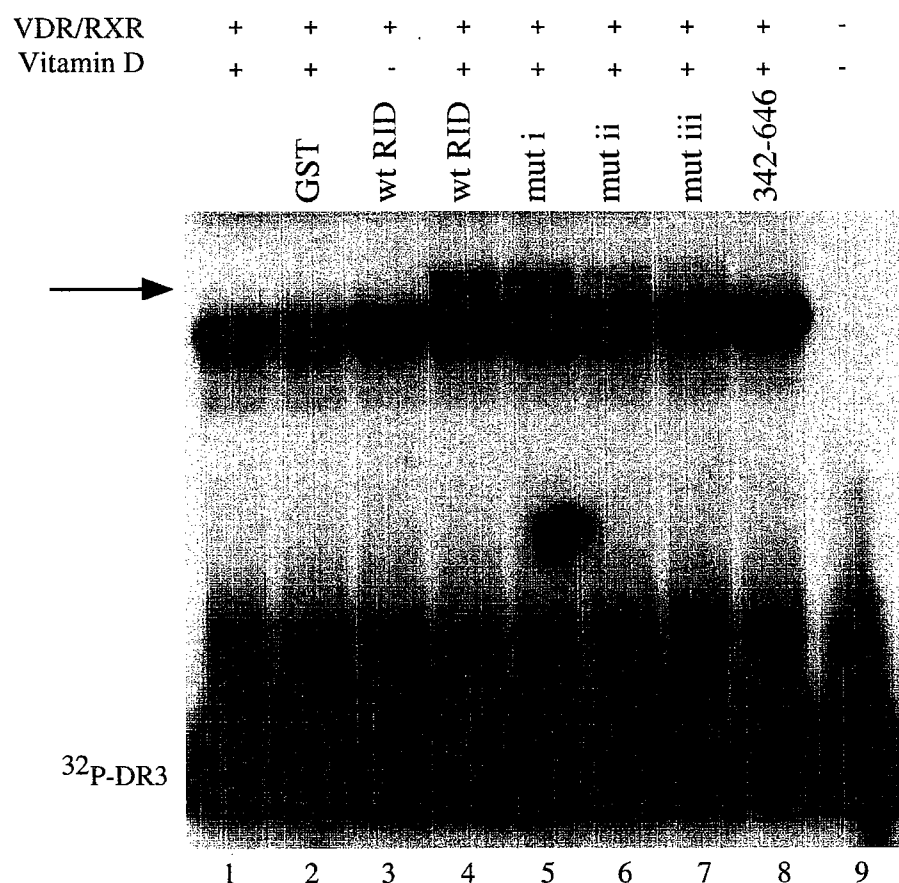


Figure 9. Mutation of RAC3 NR boxes inhibits RAC3-RID binding to DNA-bound VDR/RXR α heterodimer. Gel-shift assay using 1.5 μ L of in vitro translated VDR and RXR α , 1 μ M vitamin D₃, indicated GST fusion protein, and ³²P-DR3 probe. The arrow indicates the RID-receptor complex.

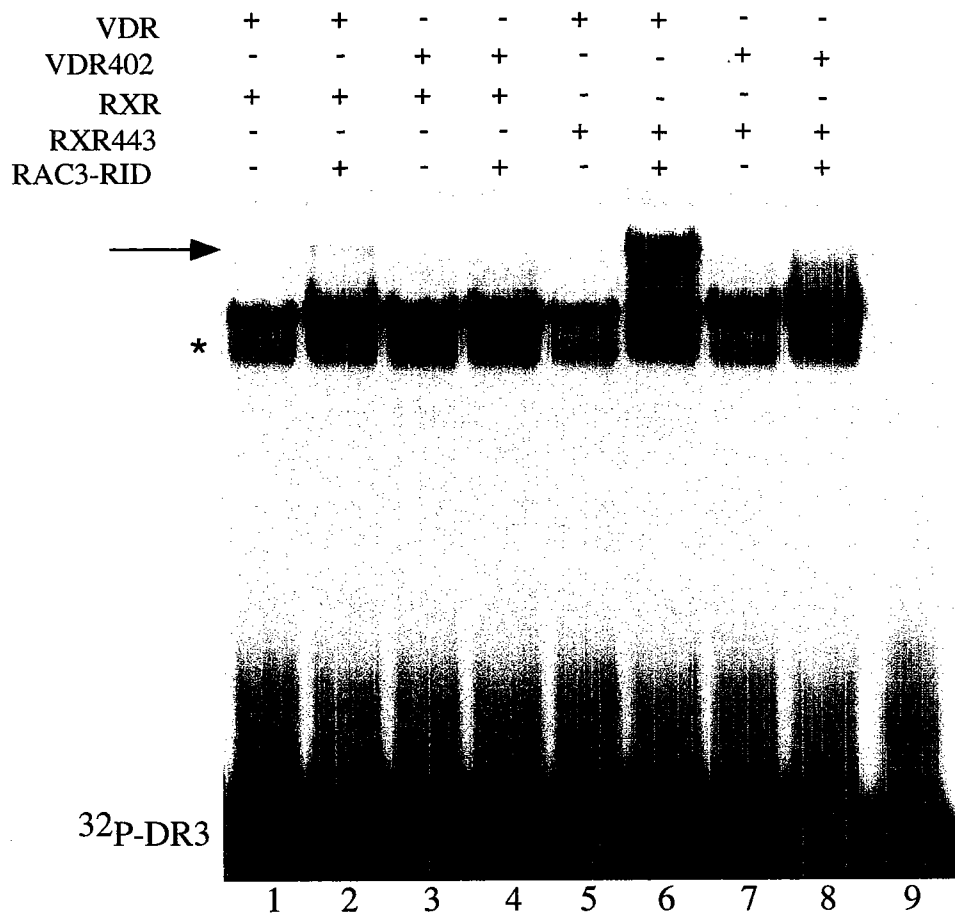


Figure 10. The RXR α AF-2 domain can interfere with RID binding to VDR/RXR α while the VDR AF-2 domain is required for the interaction. Gel-shift assay using 1.5 μ L of the indicated in vitro translated receptors, 1 μ M vitamin D₃, and ³²P-DR3 probe in presence or absence of GST-RAC3-RID. The arrow indicates the RID-receptor complex. *=non-specific band from lysate.

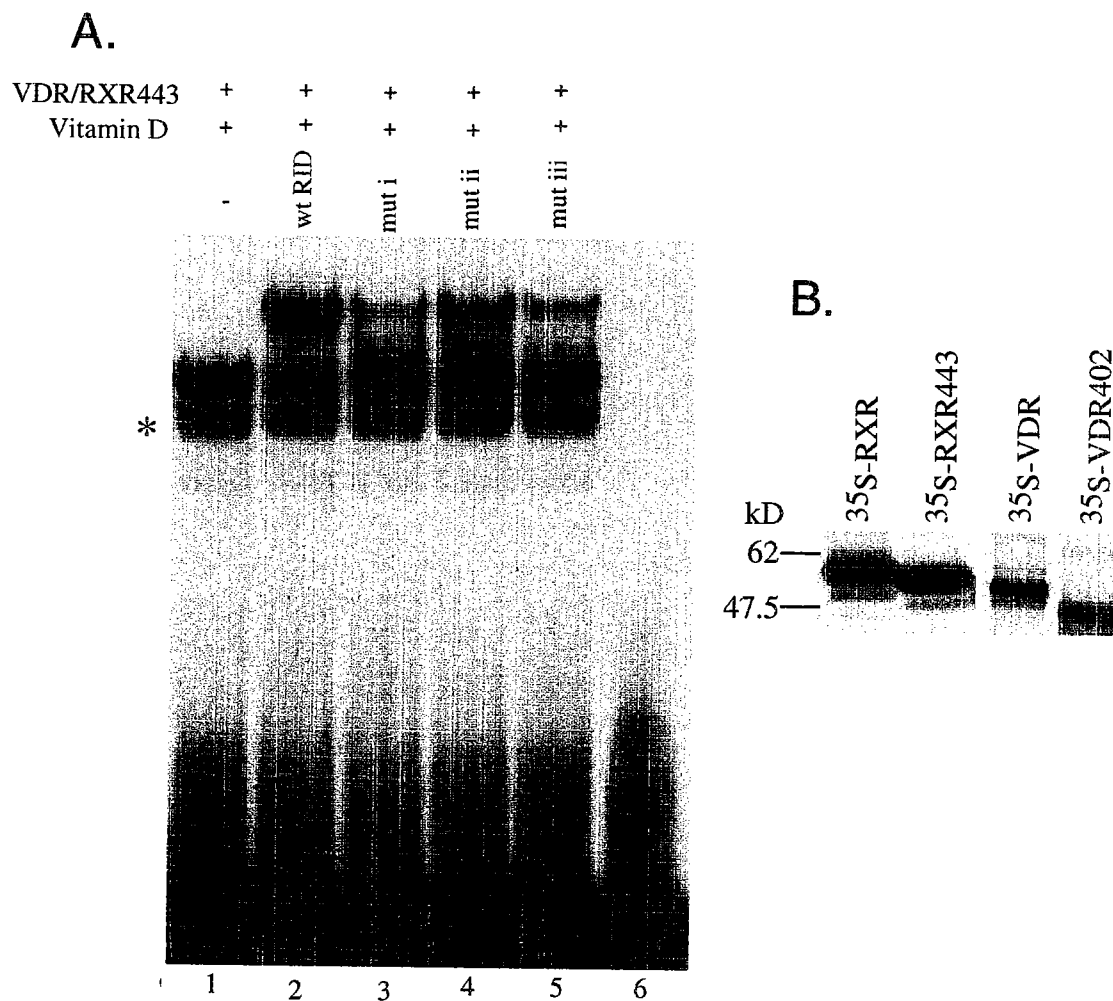


Figure 11. Distinct NR box requirements for the VDR/RXR α -443 heterodimer. (A) Gel-shift assay using 1.5 μ L of in vitro translated VDR and RXR α -443, 1 μ M vitamin D₃, ³²P-DR3 probe, and indicated GST fusion protein. *=non-specific band from lysate. (B) Autoradiograph confirming equal expression of ³⁵S-labeled receptors.

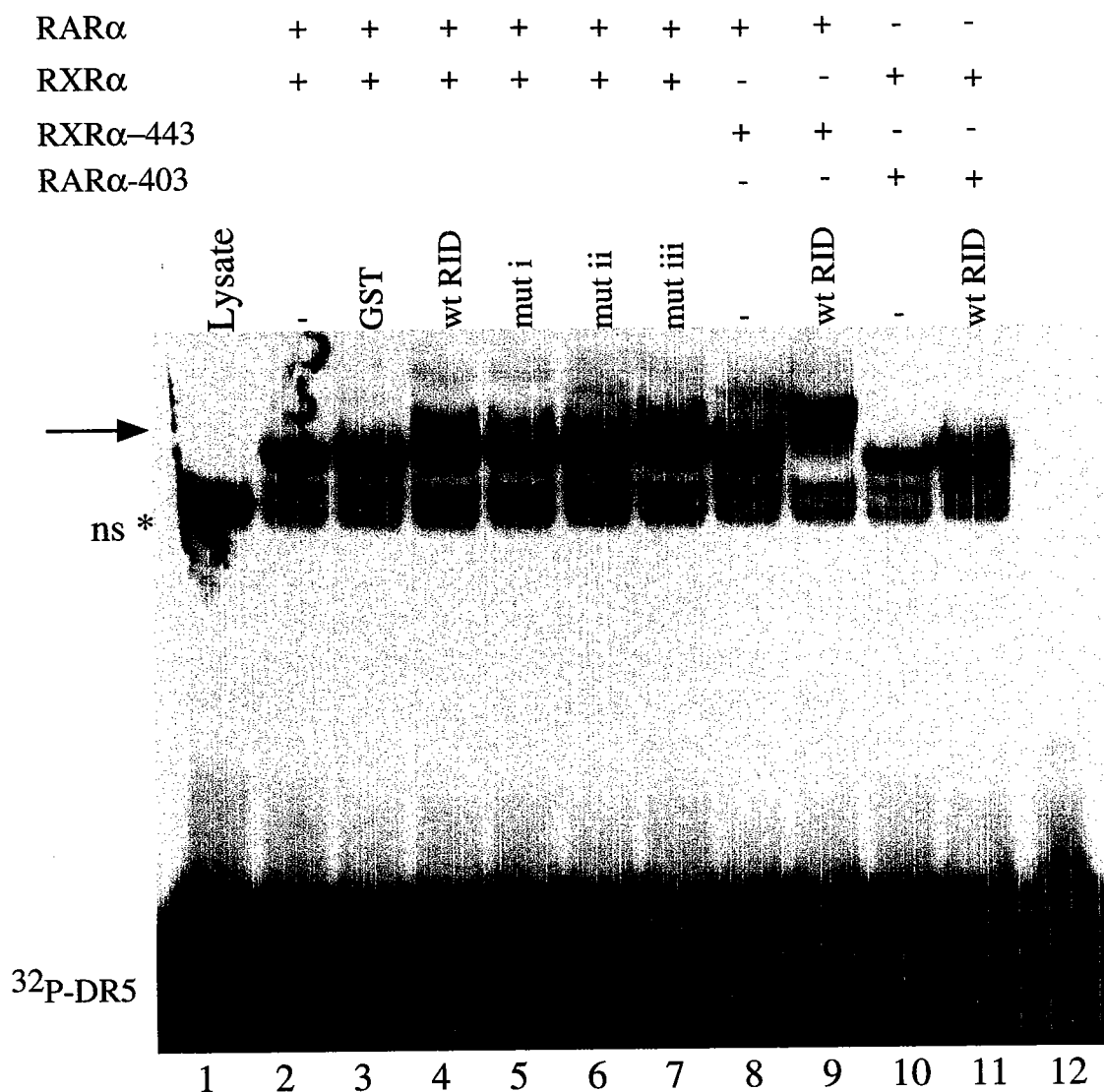


Figure 12. The RAR α /RXR α heterodimer has different NR box requirements than the VDR/RXR α heterodimer. Gel-shift assay using 1.5 μ L of in vitro translated receptors, indicated GST fusion protein, 1 μ M all-trans retinoic acid, and 32 P-DR5 probe. The AF-2 domain of RXR α also can interfere with RID binding to RAR α /RXR α while the RAR α AF-2 domain is required for this interaction. The arrow indicates the RID-receptor complex. *=non-specific band from lysate.

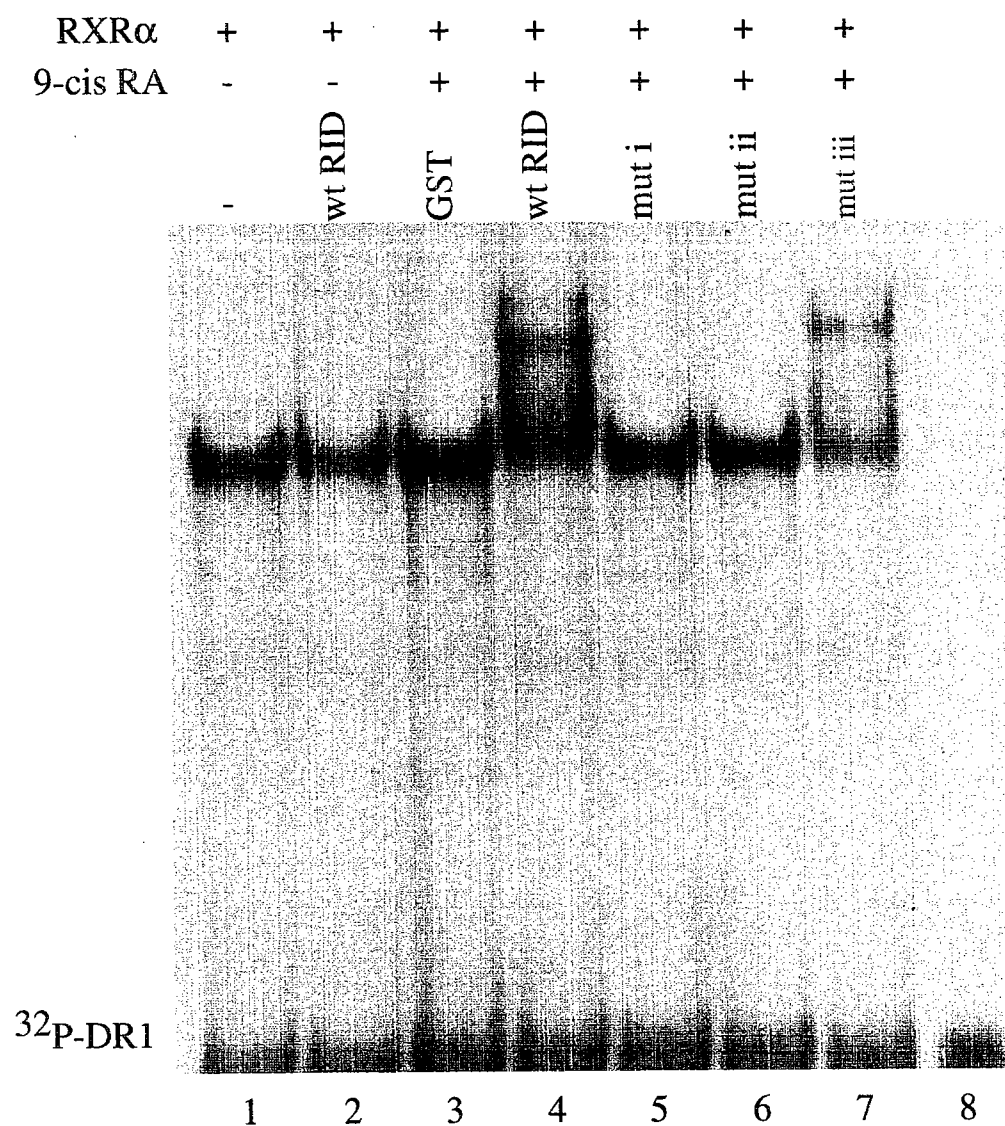


Figure 13. RAC3 NR box preferences of the RXR α /RXR α homodimer. Gel-shift assay using RXR α , ^{32}P -DR1 probe, and indicated GST fusion protein in the absence or presence of 1 μM 9-cis retinoic acid.

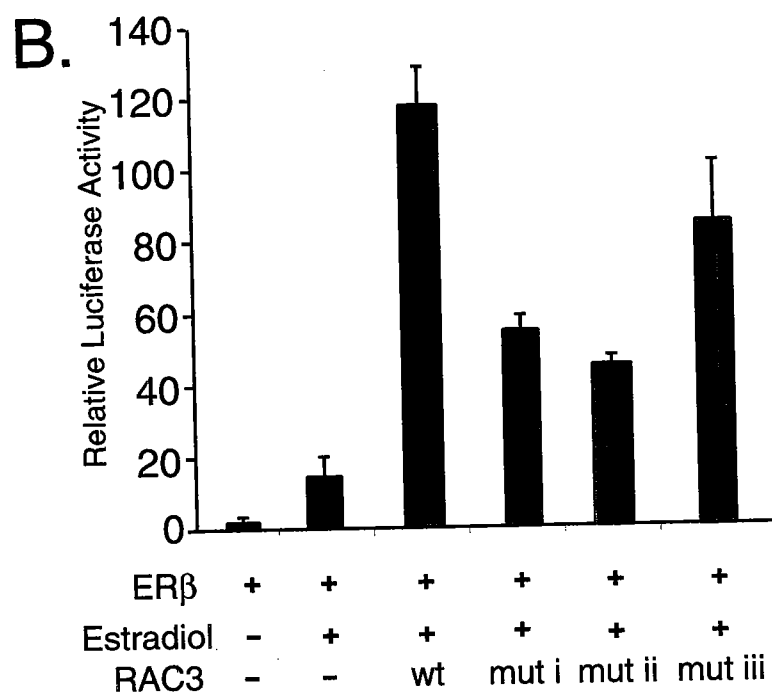
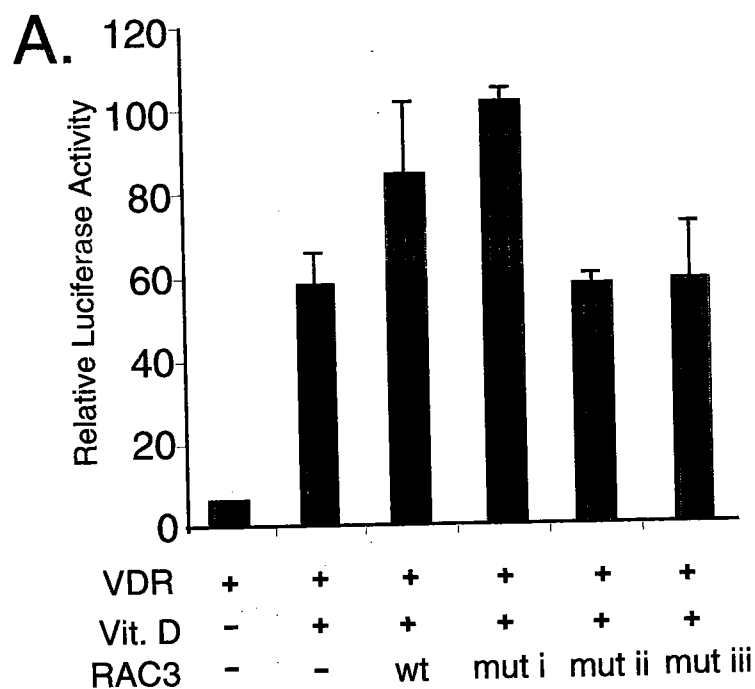


Figure 14. NR box mutation inhibits RAC3 coactivation function in vivo. (A) Transient transfection assay in CV-1 cells using VDR, VDRE-driven reporter, and indicated RAC3 construct in the absence or presence of 40nM vitamin D₃. (B) Transient transfection assay in CV-1 cells using ERβ, ERE-driven reporter, and indicated RAC3 construct in the absence or presence of 10nM 17β-estradiol.

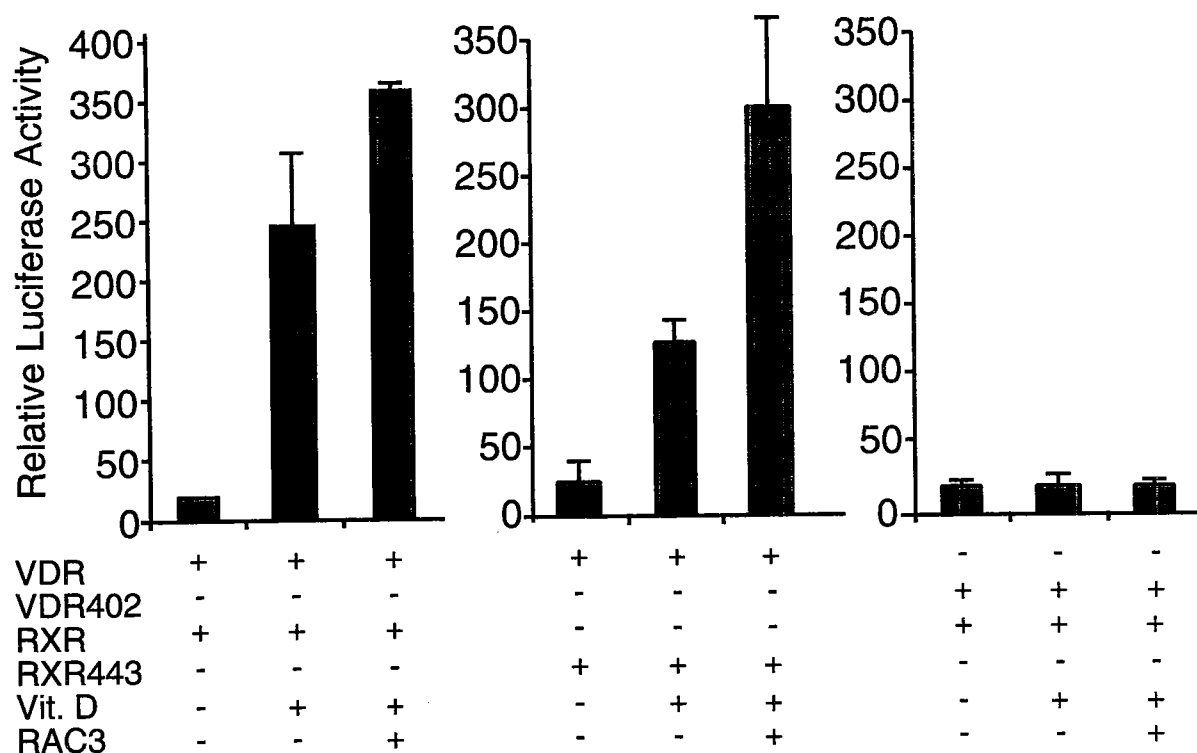


Figure 15. Comparison of RAC3 coactivation of VDR/RXR α versus VDR/RXR α -443 versus VDR-402/RXR α . Transient transfection assay in HEK293 cells using indicated receptors, a VDRE-driven reporter, and wild-type RAC3 in the absence or presence of 10nM vitamin D₃. Deletion of the RXR α AF-2 domain increases RAC3 coactivation activity while deletion of the VDR AF-2 domain abolishes transcriptional activation by the receptor.

A.

mut iv	RAC3 (1034-1038)	LDDL <u>V</u> → LDD <u>AA</u>
mut v	RAC3 (1053-1057)	LLDQ <u>L</u> → <u>AA</u> DQ <u>L</u>
mut vi	RAC3 (1078-1082)	IPE <u>L</u> V → IPE <u>AA</u>

B.

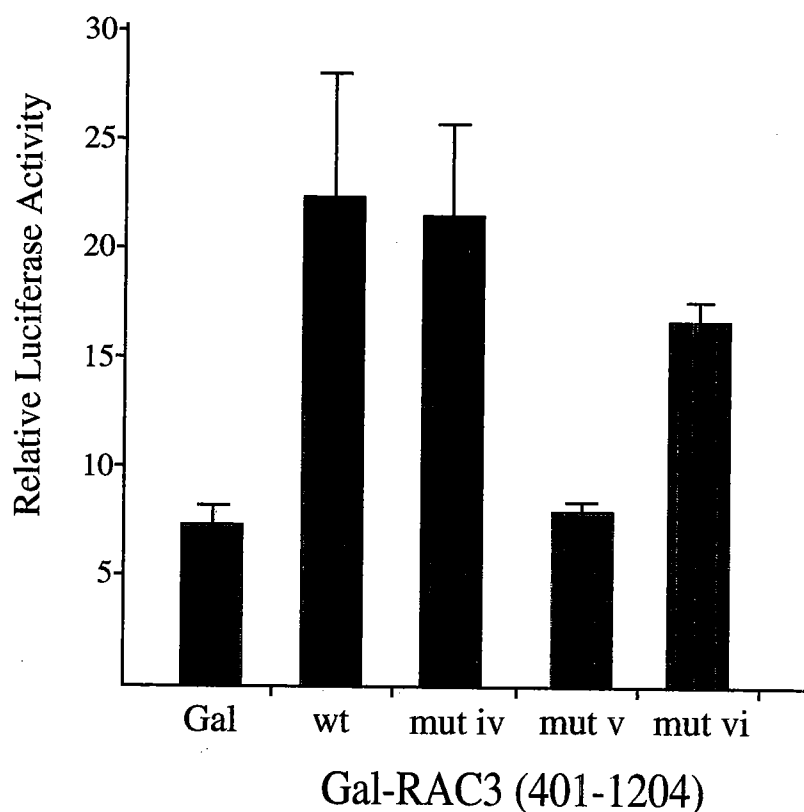


Figure 16. Mutation of NR box v inhibits RAC3 transcriptional activation. (A) Site-directed mutations of LXXLL motifs in RAC3-AD. Mutations were made in context of the Gal4-RAC3(401-1204) fusion. (B) Effect of mutations on transcriptional activation of Gal4-RAC3(401-1204) by transient transfection in HEK293 cells transfected with indicated Gal4-DBD fusion and luciferase reporter containing 4 Gal4 binding sites.

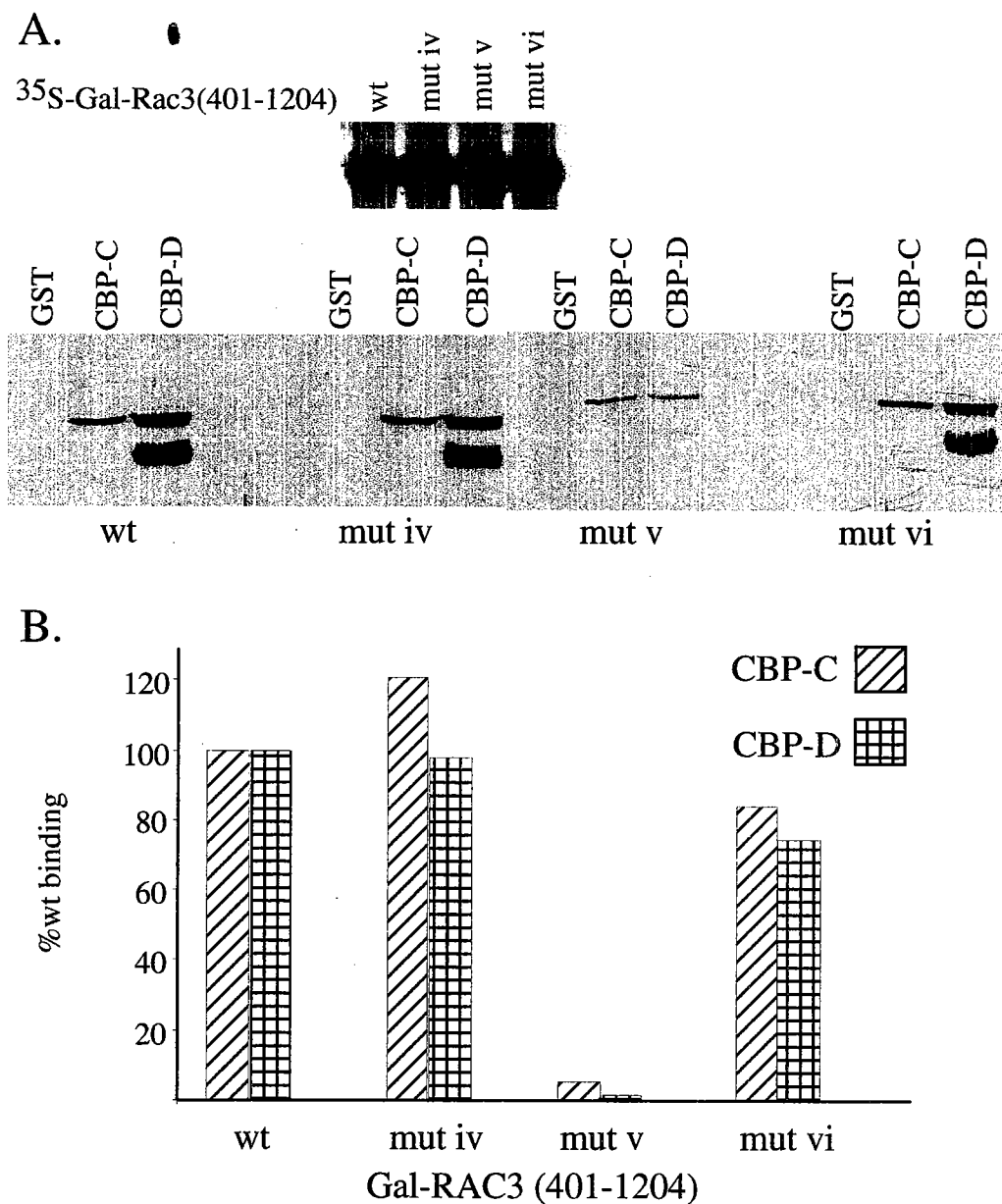


Figure 17. Mutation of RAC3 NR box v reduces interaction with CBP.

(A) Autoradiograph of ^{35}S -Gal-RAC3(401-1204) probes (top) and Far-western assay (bottom) using these probes to test for interaction with CBP-C and CBP-D fragments. (B) PhosphorImager quantitation of the data in (A). CBP-C=1921-2120 and CBP-D=2041-2240.

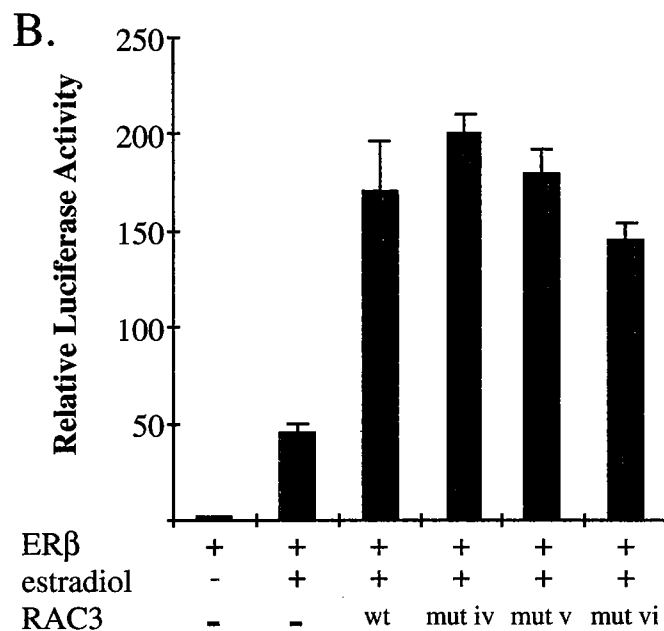
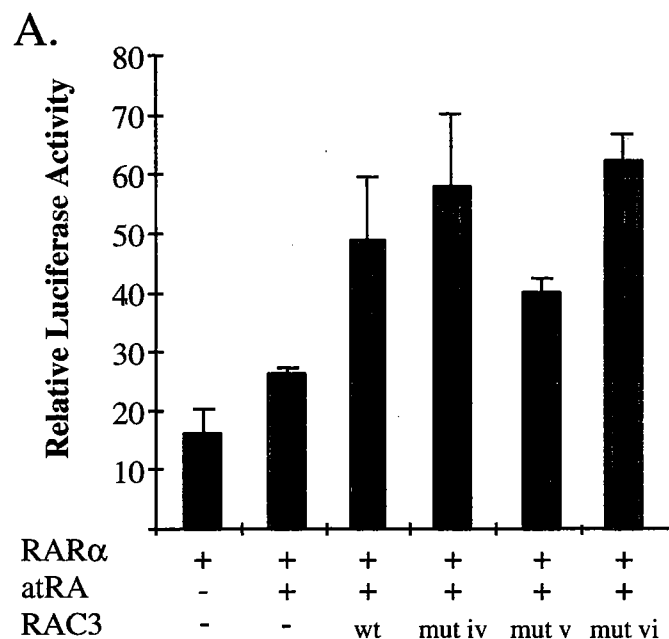


Figure 18. CBP interaction correlates with RAC3 coactivation of RAR α , but not ER β . (A) Mutation on NR box v reduces RAC3 coactivation of RAR α . Transient transfection assays in HEK293 cells. (B) Mutation of the NR boxes of the RAC3 activation domain does not affect coactivation of ER β .

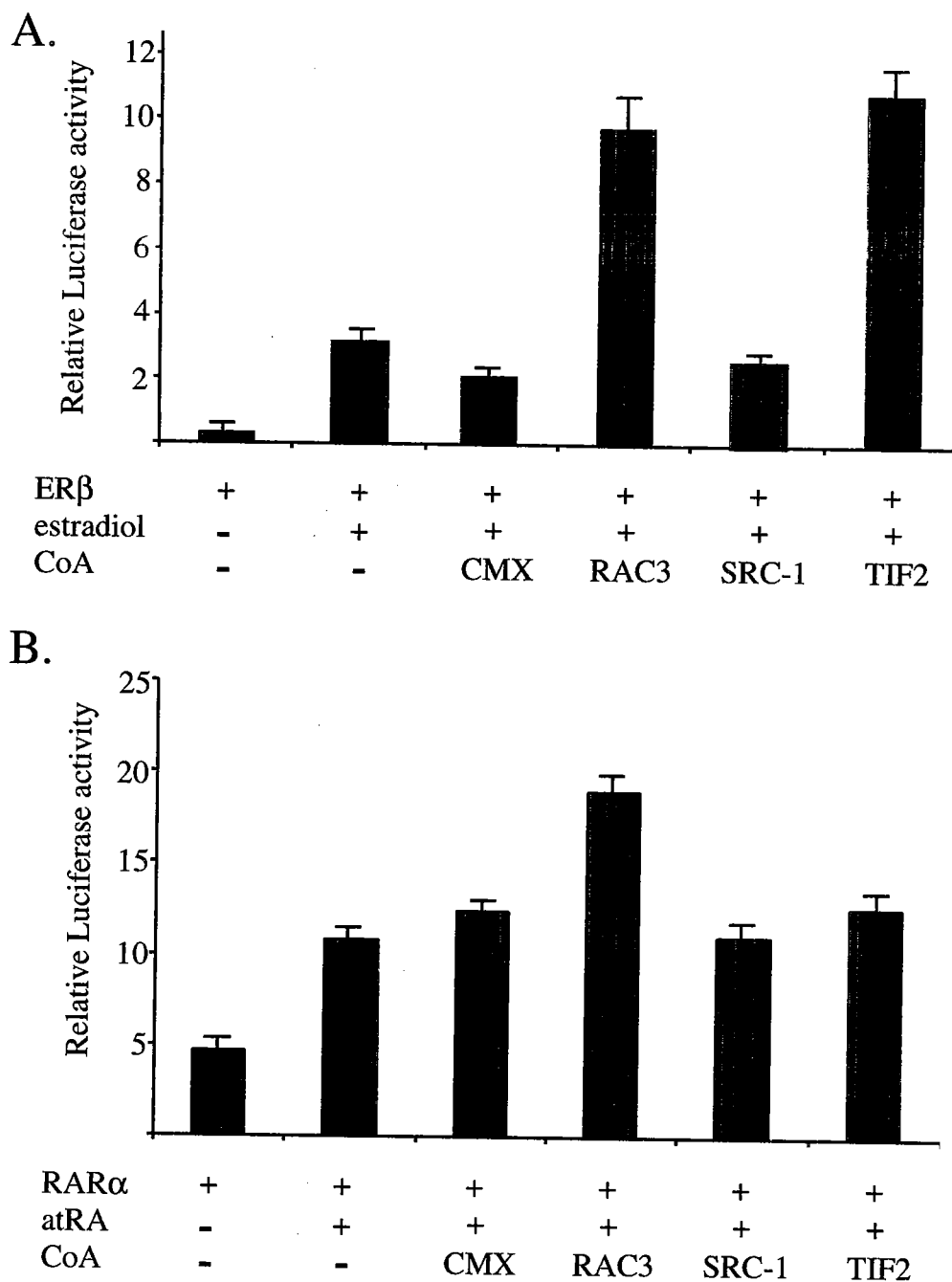
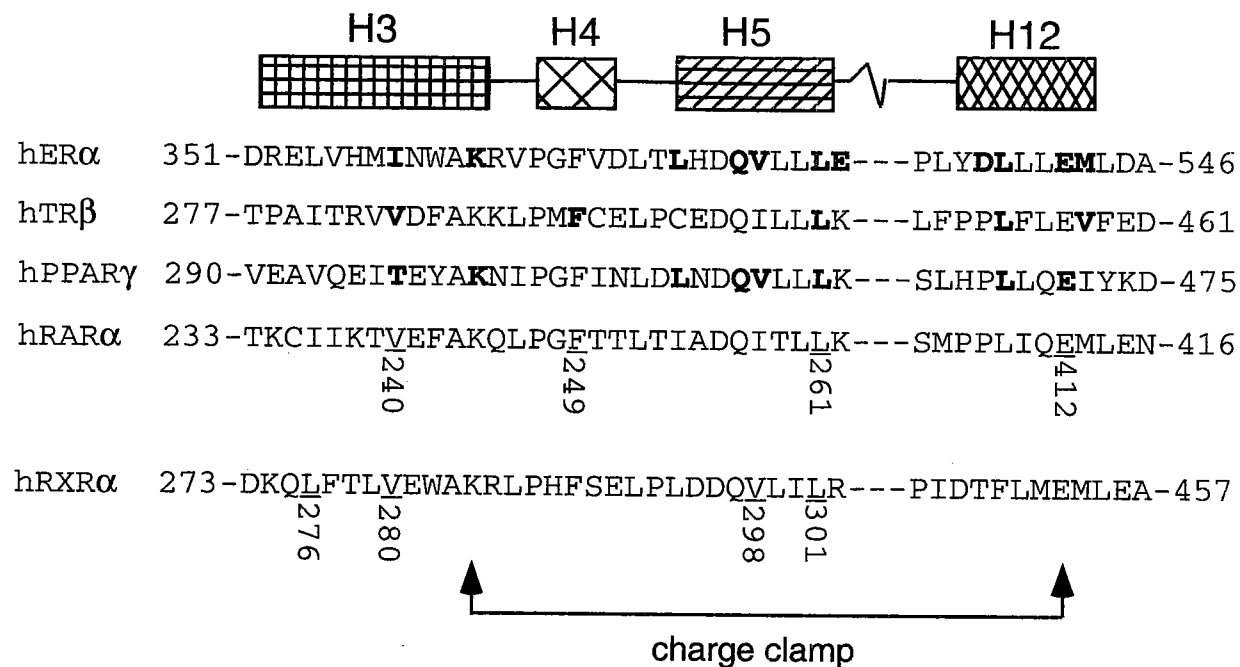


Figure 19. Receptor-specific transcriptional enhancement by SRC coactivators. (A) RAC3 and TIF2, but not SRC-1 can coactivate ER β activity. (B) Only RAC3 can coactivate RAR α activity. Transient transfection assays in HEK293 cells using ERE- or RARE-driven luciferase reporters.

Sequence Alignment of Coactivator Binding Pockets



Bold: LXXLL contact residues

Underline: mutated residues

Figure 20. Sequence alignment of the cofactor binding pocket of nuclear receptors. LXXLL contact residues are based on the crystal structures of hERα, hTRβ, and hPPARγ complexed with SRC fragments.

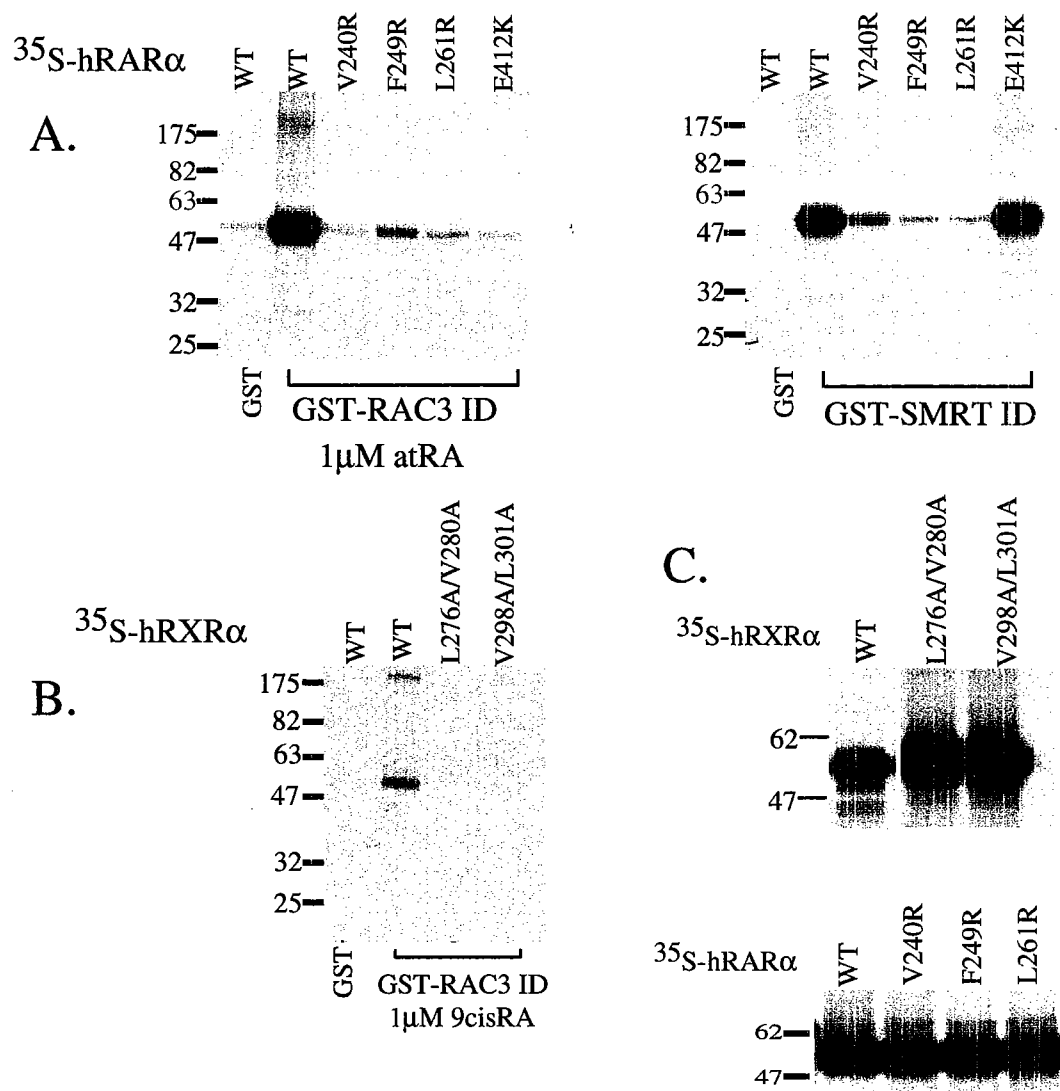


Figure 21. Mutation of the cofactor binding pocket of RAR α and RXR α inhibit interactions with coactivators and corepressors. (A) GST pull-down assay of RAR α with GST-RAC3 and GST-SMRT interacting domains. (B) GST pull-down assay of RXR α with GST-RAC3 interacting domain. (C) Autoradiograph of probes used in GST pull-down assays.

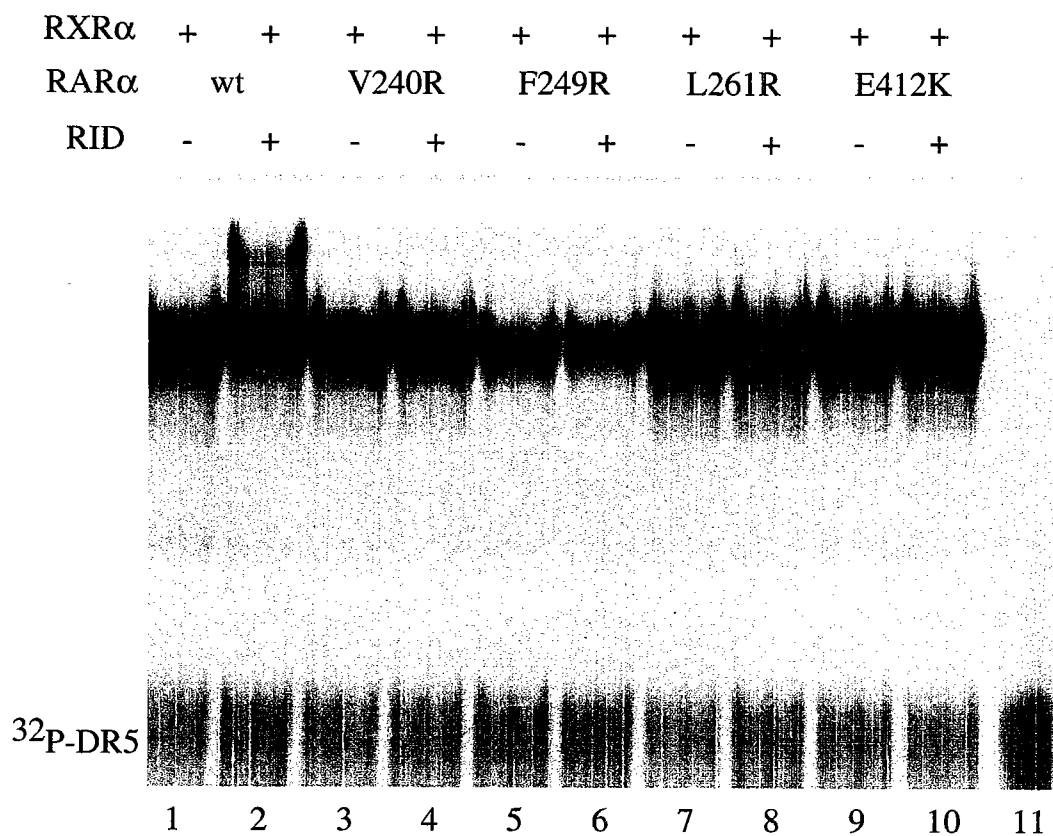


Figure 22. Mutation of the RAR α cofactor binding pocket inhibits RAC3 recruitment to the RAR α /RXR α heterodimer. Gel-shift assay using 1.5 μ L of indicated in vitro translated receptors, ³²P-DR5 probe, and 1 μ M atRA in the presence or absence of the RAC3-RID.

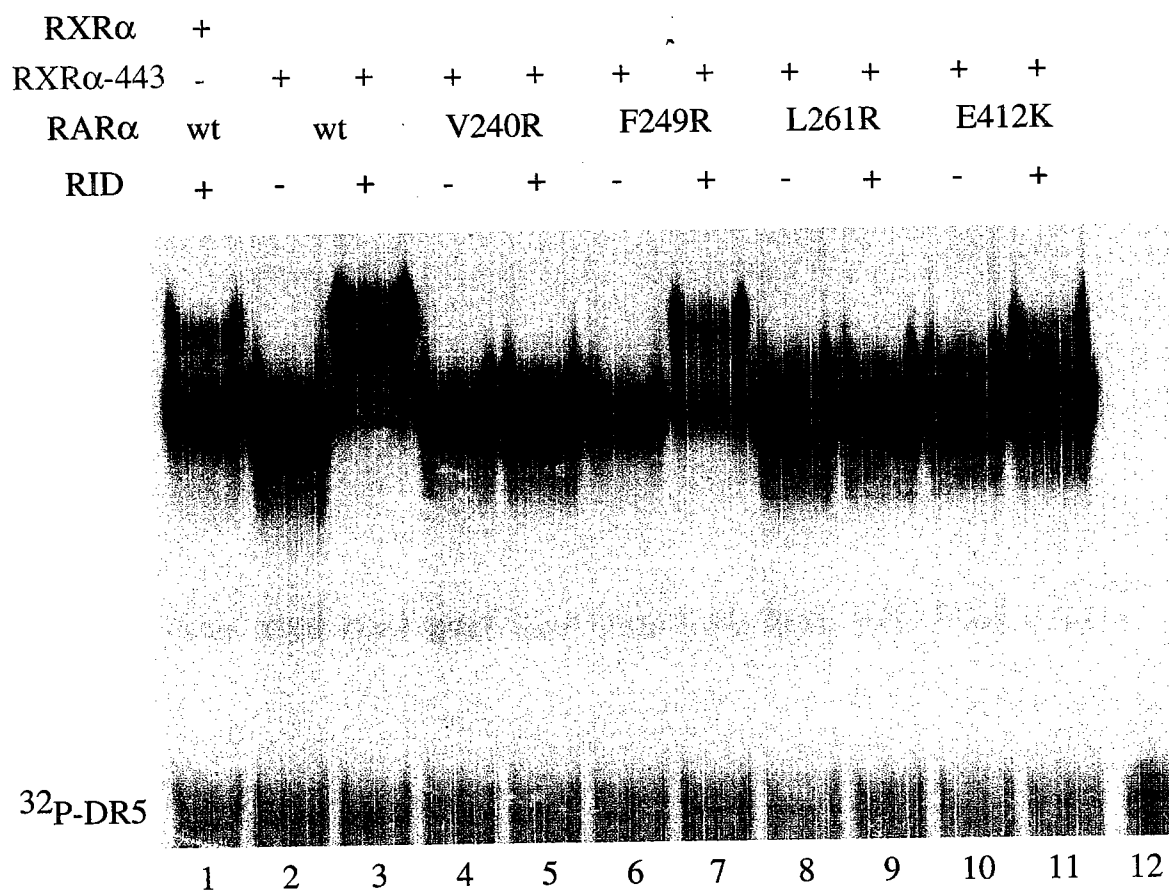


Figure 23. Mutation of the RAR α cofactor binding pocket inhibits RAC3 recruitment to the RAR α /RXR α -443 heterodimer. Gel-shift assay using 1.5 μ L of indicated in vitro translated receptors, ³²P-DR5 probe, and 1 μ M atRA in the presence or absence of the RAC3-RID.

RXR α	+	+	443	+	+	+	+	+	+	+	+
RAR α		wt	wt		V240R		F249R		L261R		E412K
SMRT-ID	-	+	-	+	-	+	-	+	-	+	-

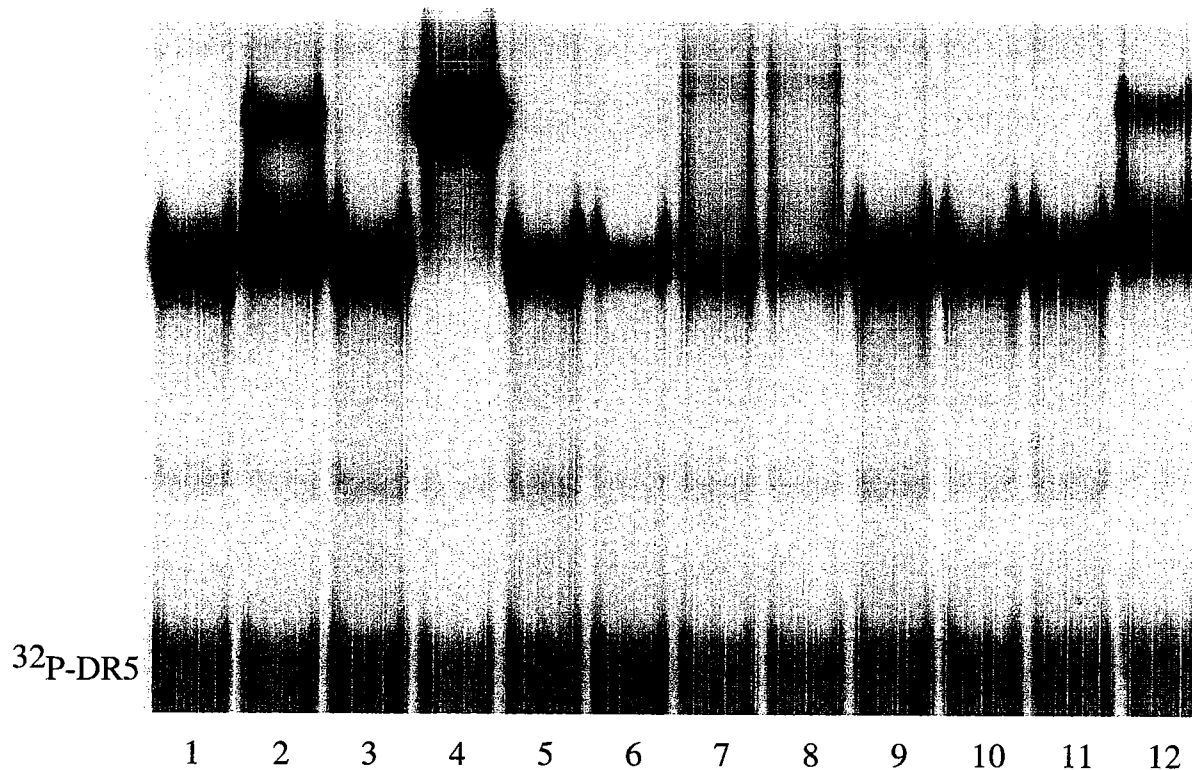


Figure 24. Mutation of the RAR α cofactor binding pocket inhibits recruitment of SMRT to the RAR α /RXR α heterodimer in the absence of hormone. Gel-shift assay using 1.5 μ l of each of the indicated in vitro translated receptors and a 32 P-DR5 probe in a binding reaction in the absence or presence of purified GST-SMRT-ID (982-1495).

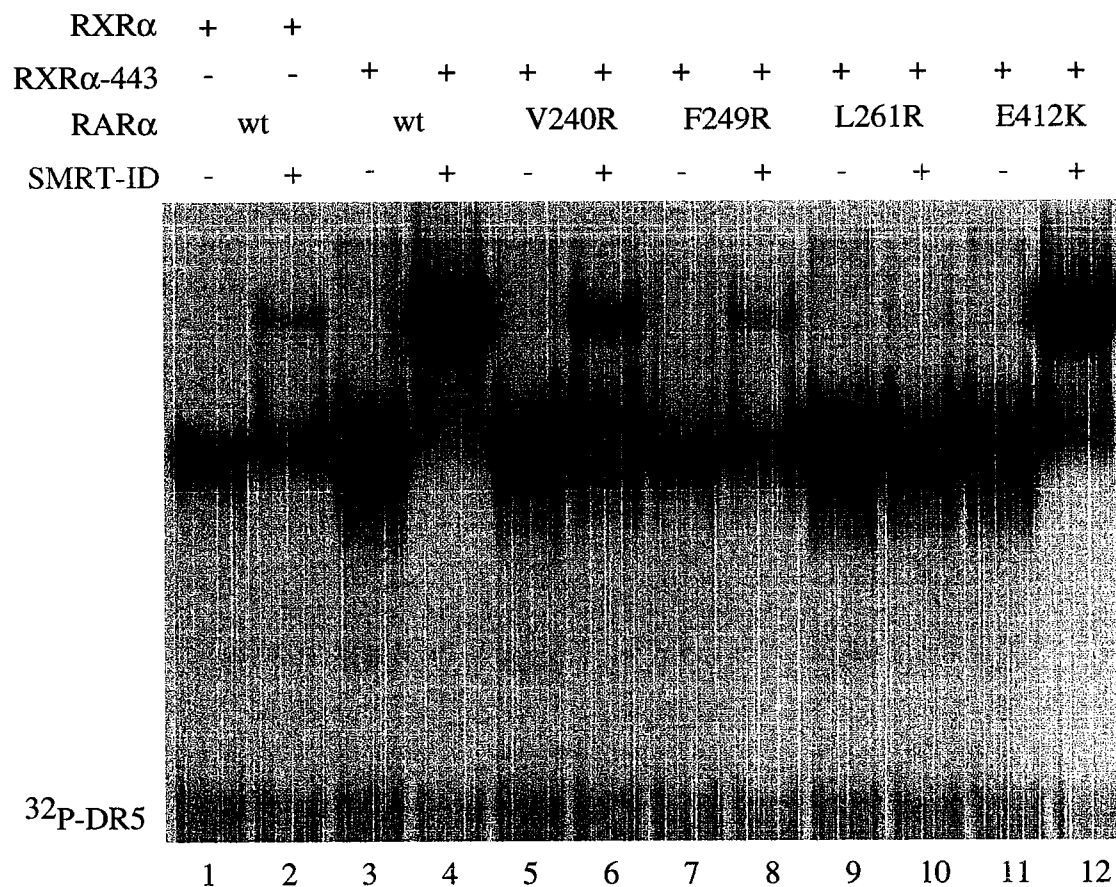


Figure 25. Mutation of the RAR α cofactor binding pocket inhibits SMRT recruitment to the unliganded RAR α /RXR α -443 heterodimer. Gel-shift assay using 1.5 μ L of each indicated in vitro translated receptor and a ³²P-DR5 probe in the presence or absence of GST-SMRT-ID (982-1495).

RAC3-ID	-	+	-	+	-	+	-	+	+	+
RXR α	+	+	+	+	m1	m1	m2	m2	m1	m2
RAR α	+	+	V240R		+	+	+	+	V240R	

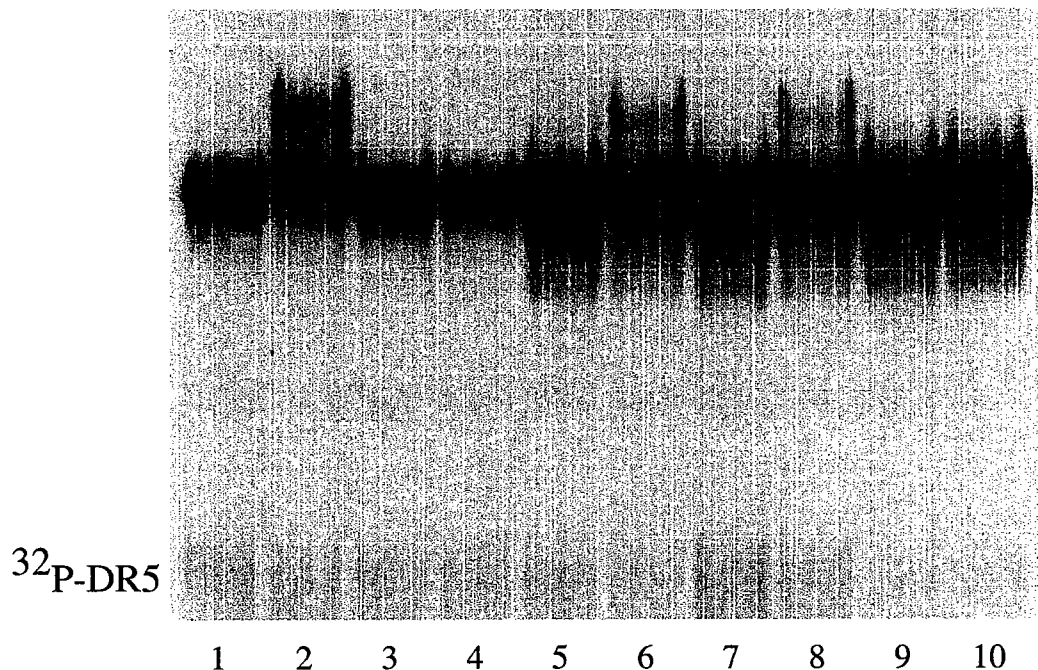


Figure 26. Effect of RXR α cofactor binding pocket mutation on RAC3 recruitment to RAR α /RXR α heterodimer at DR5 element. Gel-shift assay using 1.5 μ L of each indicated in vitro translated receptor, 1 μ M all-trans retinoic acid, and a 32 P-DR5 probe in the presence or absence of the RAC3-RID. m1=L276A/V280A, m2=V298A/L301A

RAC3-ID	-	+	-	+	-	+	-	+
RXR α	+	+	-	-	-	-	m2	m2
RXR α -443	-	-	+	+	m2	m2	-	-
RAR α	+	+	+	+	+	+	+	+

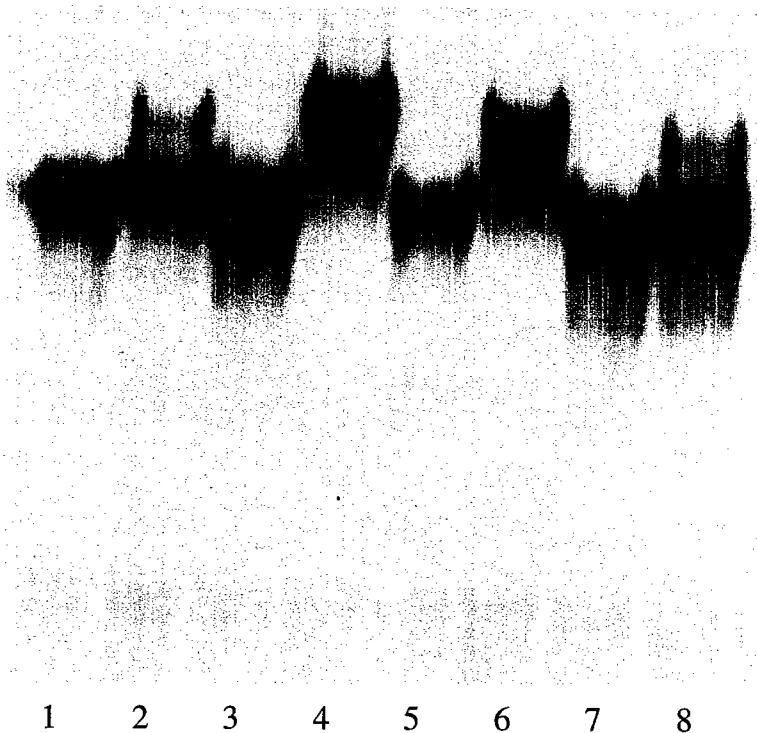


Figure 27. Mutation of the RXR α cofactor binding pocket has little effect on RAC3 binding to the RAR α /RXR α or RAR α /RXR α -443 heterodimers. Gel shift assay using 1.5 μ L of each indicated in vitro translated receptor, a 32 P-DR5 probe, and 1 μ M all-trans RA in the presence or absence of the RAC3-RID. m2=V298A/L301A

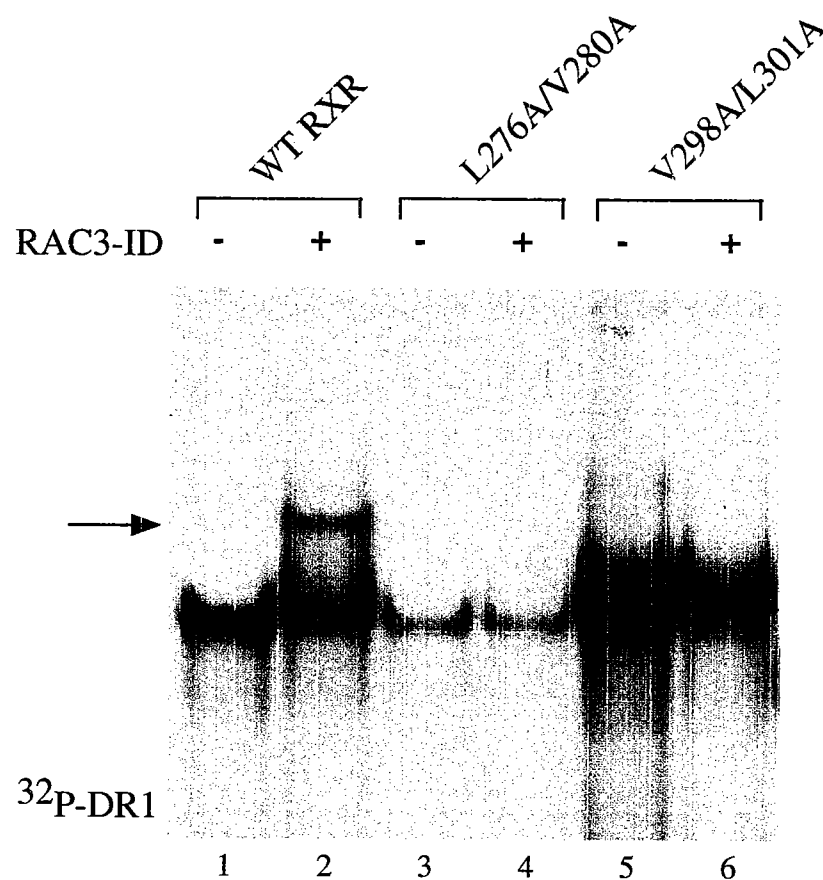


Figure 28. Mutation of the cofactor binding pocket of RXR α abolishes recruitment of RAC3 to the RXR α homodimer. Gel-shift assay using 1.5 μL of in vitro translated wild-type or mutant RXR α , 1 μM 9-cis retinoic acid, and a $^{32}\text{P-DR1}$ probe in presence or absence of the RAC3-RID. The arrow indicates the RID-receptor complex.

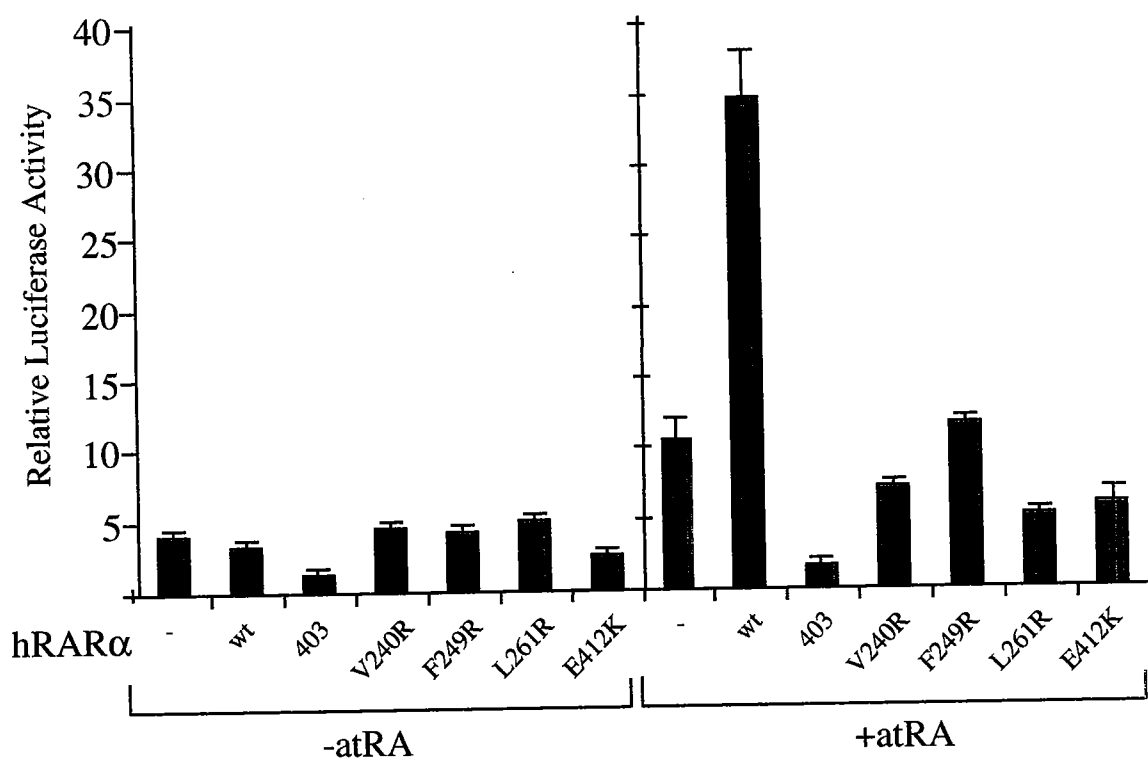


Figure 29. Transcriptional activity of RAR α cofactor binding pocket mutants. Transient transfection assay in HEK293 cells of wild-type or mutant RAR α and RARE-driven luciferase reporter in absence (left) or presence (right) of 50nM all-trans retinoic acid.

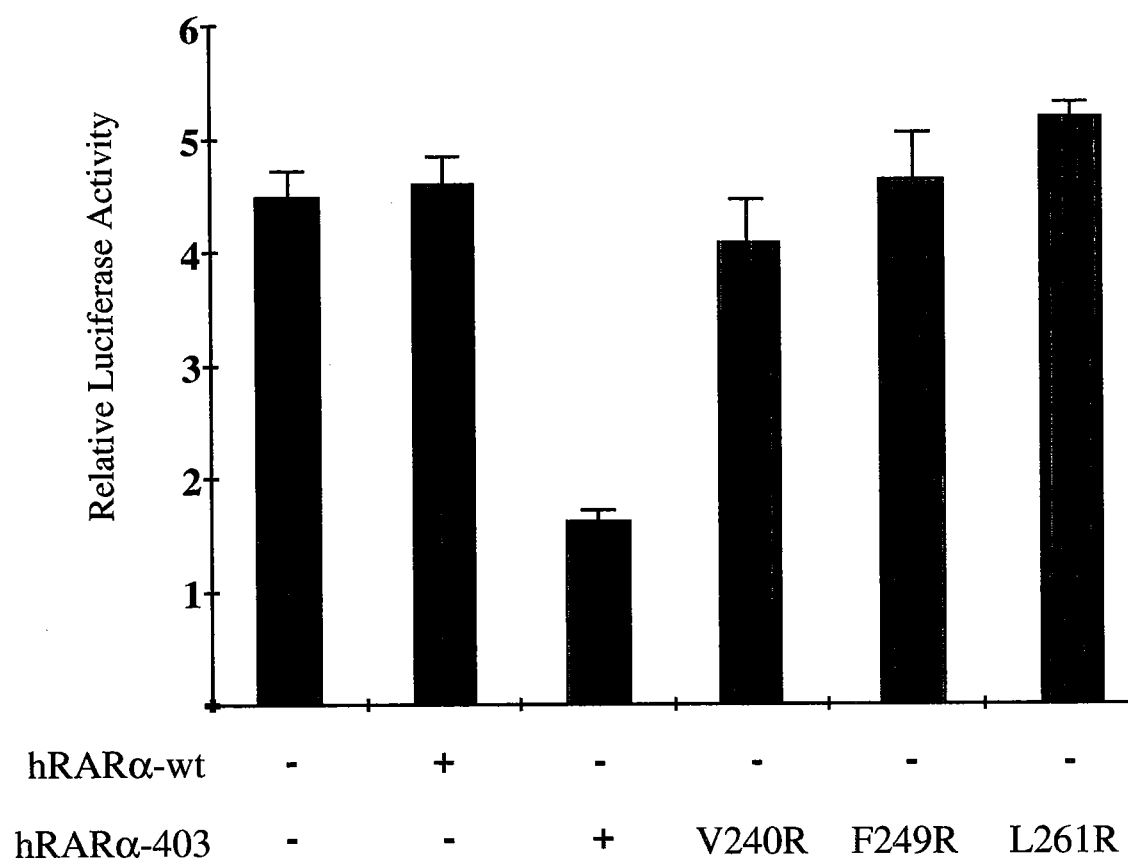


Figure 30. Mutation of the cofactor binding pocket abolishes transcriptional repression by RARα-403. Transient transfection assay in HEK293 cells of indicated receptor and RARE-driven luciferase reporter.

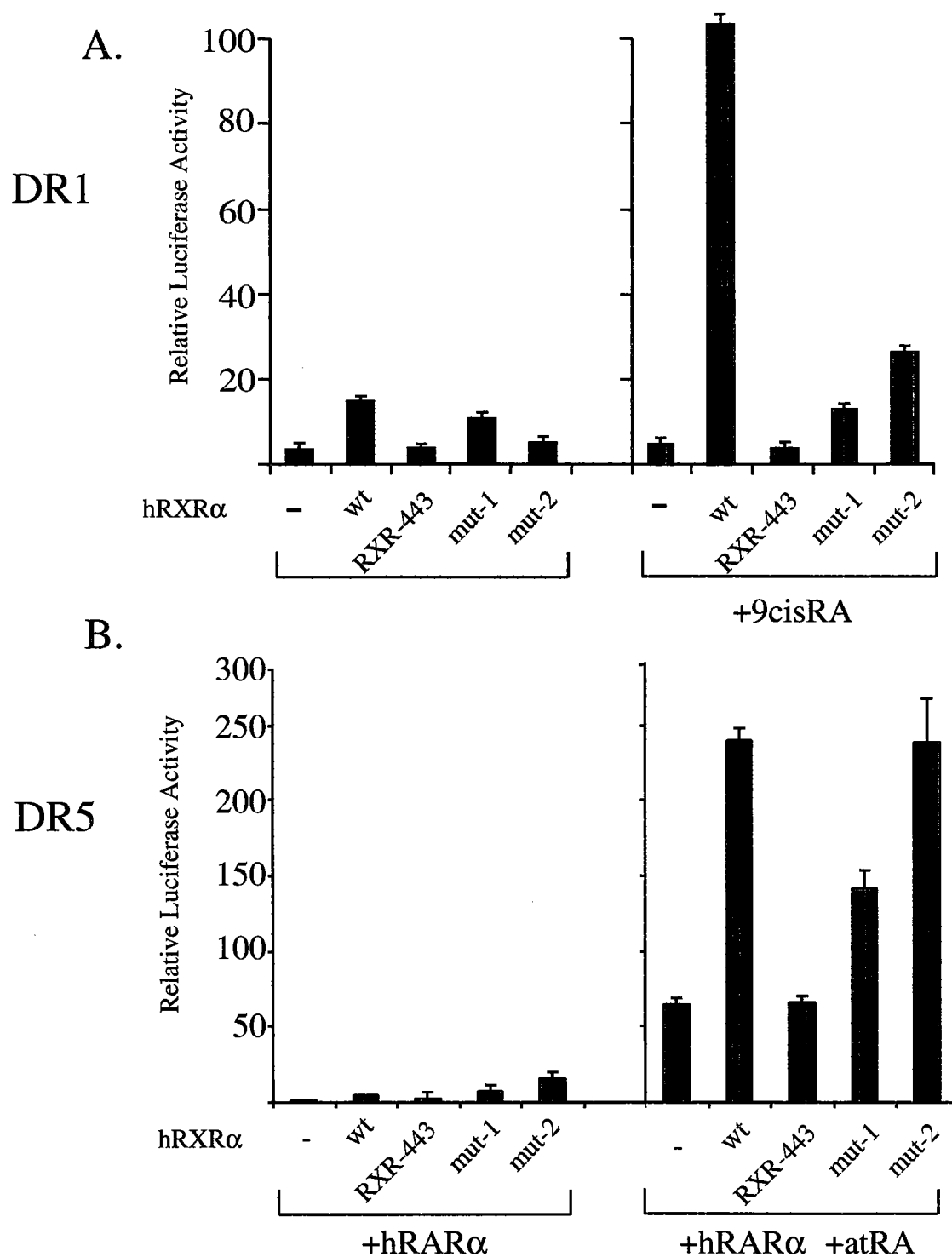


Figure 31. Mutation of the RXR α cofactor binding pocket has different effects on transcriptional activity at a DR1- versus DR5-driven reporter. (A) Transient transfection assay in HEK293 cells of indicated receptor and DR1-driven luciferase reporter in absence (left) or presence(right) of 100nM 9cis retinoic acid. (B) The same assay was repeated using a DR5-driven reporter and hRAR α in absence (left) or presence(right) of 100nM all-trans acid. mut-1=L276A/V280A, mut-2=V298A/L301A

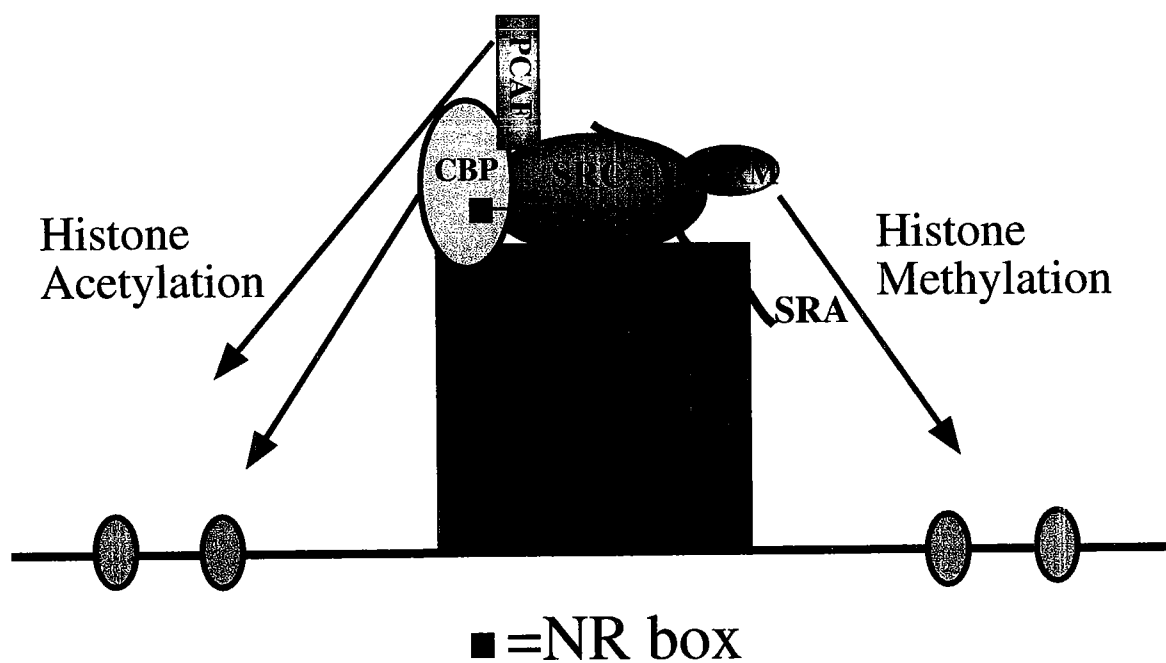
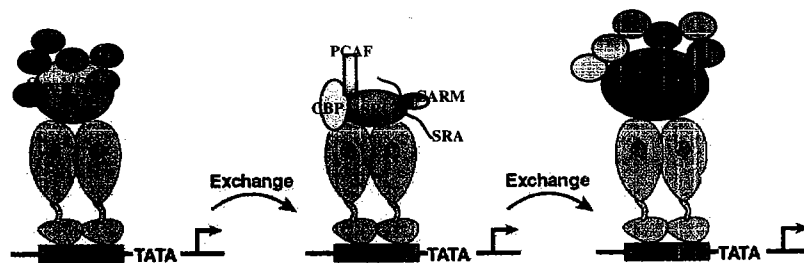
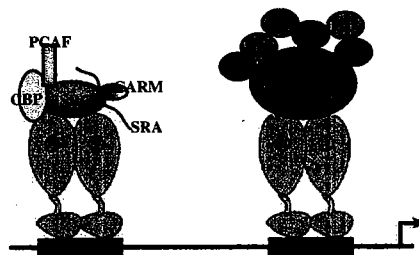


Figure 32. Model of SRC coactivator function. An SRC coactivator is recruited to a DNA-bound nuclear receptor dimer via the LXXLL motifs. Subsequently, SRC is able to enhance transcriptional activation by the receptor by recruiting additional coactivators such as CBP/p300, PCAF, SRA, and/or CARM1, which modify chromatin via histone acetylation and histone methylation.

Sequential



Cooperative



Parallel

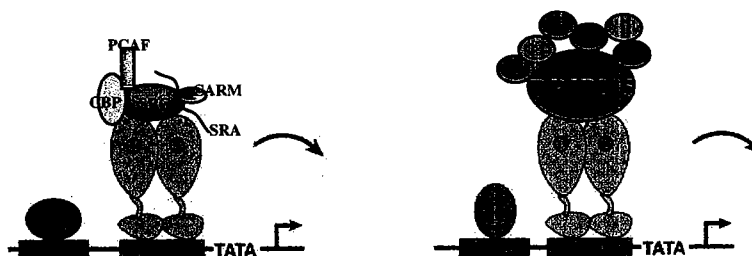


Figure 33. Models of Multiple Coactivator Complexes. One of the unanswered questions concerning nuclear receptor function is the mechanism by which multiple coactivator complexes are coordinated into transcriptional activation by the receptor. The above models, which are described in the text, provide several hypotheses.

CHAPTER VIII

REPRINTS

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Differential Mechanisms of Nuclear Receptor Regulation by Receptor-associated Coactivator 3*

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Steroid and nuclear receptor coactivators (NCoAs) have been implicated in the regulation of nuclear receptor function by enhancing ligand-dependent transcriptional activation of target gene expression. We have previously isolated receptor-associated coactivator 3 (RAC3), which belongs to the steroid receptor coactivator family. In this study, we investigated the differential mechanisms by which RAC3 interacts with and modulates the transcriptional activity of different nuclear receptors. We found that the vitamin D receptor (VDR) and estrogen receptor β interact with different α -helical LXXLL motifs of RAC3. Peptides corresponding to these motifs have diverse affinities for the VDR and estrogen receptor β , and mutation of specific motifs differentially impairs the ability of RAC3 to interact with these receptors *in vitro*. Consequently, these mutations inhibit the enhancement of transcriptional activation by these receptors *in vivo*. Furthermore, we found that the activation function-2 (AF-2) domain of the retinoid X receptor interferes with RAC3 binding to a DNA-bound VDR/retinoid X receptor (RXR) heterodimer, whereas the VDR AF-2 domain is required for this interaction. These results suggest a receptor-specific binding preference for the different LXXLL motifs of RAC3, which may provide flexibility for RAC3 to differentially regulate the function of different nuclear receptors.

The vitamin D receptor (VDR)¹ and estrogen receptor β (ER β) belong to the steroid/thyroid hormone receptor superfamily, which is a large class of ligand-dependent transcription factors that plays critical roles in regulating genes involved in a wide array of biological processes, including development and homeostasis (1). This superfamily can be divided into three subgroups. The ER β is a Type I receptor, which also includes receptors for steroids such as progestins, androgens, glucocorticoids, and mineralocorticoids. These receptors are coupled to

heat shock proteins and sequestered to the cytoplasm in the absence of ligand. Upon hormone binding, they dissociate from the heat shock proteins, homodimerize, and translocate to the nucleus where they bind to cognate response elements consisting of palindromic repeats. The VDR is a Type II receptor like those for thyroid hormone (TR) and all-trans retinoic acid (RAR). These receptors are strictly nuclear and form heterodimers with the receptor for 9-cis retinoic acid (RXR). They also bind constitutively to response elements consisting of direct repeats (DRs). A third class of nuclear receptors is the orphan receptors, so-called because endogenous ligands for these proteins are currently unknown.

Most members of the nuclear receptor superfamily share a common domain structure. The N terminus contains the variable A/B region, which also includes the ligand-independent AF-1 activation domain. The highly conserved DNA binding domain and the C-terminal ligand binding domain (LBD) follow this region. The LBD contains the ligand-dependent AF-2 activation domain and also mediates dimerization of nuclear receptors. In the absence of ligand, nuclear receptors are able to repress basal transcription via functional interactions with the nuclear receptor corepressors SMRT and NCoR (2, 3). SMRT and NCoR are found in complexes with the corepressor mSin3 and the histone deacetylase HDAC1, suggesting that transcriptional repression by nuclear receptors may involve histone deacetylation (4–6). Ligand binding triggers the release of these corepressors and subsequent recruitment of coactivators through a drastic conformational change in the AF-2 domain of the receptor. Structural studies have demonstrated that helix 12, which contains the AF-2 domain, projects away from the LBD in the unbound RXR structure, but rotates nearly 180° to pack tightly against the LBD upon hormone binding in the RAR, TR, and ER (7–10). This conformational change, together with induced changes in helices 3–5, is believed to facilitate interactions of the receptor with coactivators (11–16).

Coactivators recruited by ligand-bound nuclear receptors include members of the SRC family of coactivators such as SRC1 (also known as NCoA-1), TIF2/GRIP1 (also known as SRC2 or NCoA-2), and RAC3/ACTR/pCIP/AIB1 (also known as SRC3 or NCoA-3) (reviewed in Refs. 17 and 18). SRC family members share an N-terminal basic helix-loop-helix/PAS-A/PAS-B domain of unknown function, centrally located receptor interaction domain, and C-terminal transcriptional activation domain. These cofactors interact with receptors in a hormone- and AF-2-dependent manner and enhance transcriptional activation by nuclear receptors. Both coactivators and receptors also have been demonstrated to interact with the general transcriptional activators CBP/p300 and PCAF (19–26), suggesting that a large multi-protein complex is assembled at the target gene promoter to activate transcription. Furthermore, several coactivators, including SRC1, ACTR, PCAF, and CBP/p300, possess

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¹ The abbreviations used are: VDR, vitamin D receptor; RXR, retinoid X receptor; RAR, retinoic acid receptor; ER β , estrogen receptor β ; RAC3, receptor-associated coactivator 3; SRC, steroid receptor coactivator; RID, receptor-interacting domain; AF-2, activation function-2 domain; NR box, nuclear receptor interacting box; DR, direct repeat; LBD, ligand binding domain; GST, glutathione S-transferase; NCoA, nuclear receptor coactivator; PAS, Per-Arnt-Sim domain; CBP, CREB-binding protein; PCAF, p300/CBP-associated factor; ERE, estrogen response element; TR, thyroid hormone.

intrinsic histone acetylation activity, which disrupts nucleosomes (21, 27–30). Therefore, the mechanism by which nuclear receptors activate transcription may entail the recruitment of a coactivator complex via the AF-2 domain that can modify chromatin structure, thereby facilitating access to the promoter by the general transcription machinery.

Intriguingly, members of the SRC family of coactivators have been found to contain several conserved motifs, termed NR boxes, with the consensus sequence LXXLL, where X is any amino acid (31). Motifs within the receptor-interacting domain and transcriptional activation domains of SRC1 and TIF2 have been demonstrated to mediate interactions with liganded nuclear receptors and CBP/p300, respectively (23, 32). Crystallographic and protein structure prediction analyses have indicated that these motifs form amphipathic α -helices with the leucine residues comprising a hydrophobic surface on one face of the helix (11, 12, 14, 24). The helical motif is able to interact with the AF-2 domain of the liganded receptor via a hydrophobic groove made up of residues from receptor helices 3, 4, 5, and 12 that is the result of the conformational change induced by hormone binding (11, 14, 16). Mutational analyses of the NR boxes of SRC1 and TIF2/GRIP1 have also uncovered a receptor-specific code of interaction, where different nuclear receptors require different NR boxes to interact with the coactivator (32–34). These studies indicate that flanking residues outside the NR box may also be important to nuclear receptor-coactivator interactions.

In this study, we investigate the mechanisms by which RAC3 regulates the function of the VDR and ER β , for little is known concerning the regulation of these receptors by SRC coactivators, particularly RAC3. These analyses reveal receptor-specific interactions in which the VDR and ER β interact with different surfaces of RAC3. We demonstrate different preferences of these receptors for specific NR boxes of RAC3 and that single mutations in these LXXLL motifs are able to severely impair the ability of RAC3 to interact with and, thus, coactivate the VDR and ER β . In analyzing the requirement of nuclear receptor AF-2 domains, we observe that the AF-2 domain of RXR can inhibit RAC3-RID binding to the DNA-bound VDR/RXR heterodimer, whereas the AF-2 domain of VDR is required for this interaction. These data add a new level of complexity to the regulation of nuclear receptor activity by SRC coactivators and suggest that different classes of nuclear receptors may be regulated by RAC3 via different mechanisms.

EXPERIMENTAL PROCEDURES

Far Western Analysis—Far Western assays were carried out as described (20). Briefly, GST fusion proteins were expressed in DH5 α cells and purified with glutathione-agarose beads (Amersham Pharmacia Biotech). Purified proteins were then separated by SDS-polyacrylamide gel electrophoresis and electroblotted onto a nitrocellulose membrane. Proteins were denatured with 6 M guanidine hydrochloride and renatured by the stepwise dilution of guanidine hydrochloride. Membranes were then blocked and hybridized overnight with 35 S-labeled protein. The membrane was washed, and bound probe was detected by autoradiography. 35 S-labeled probes were generated by Quick-coupled *in vitro* transcription/translation (Promega). For peptide competition experiments, the given concentration of peptide was added to the probe 10 min before hybridization with the membrane. Peptide sequences were as follows: NR box i peptide (LESKGHKLLQLLTSSDDRGHSSL), NR box ii peptide (LQEKHRLHKLLQNGNSP), NR box iii peptide (KKKENNALLRYLLDRDD), control peptide (GSGSATATLYENKPRPYIL). Radioactive bands were quantified by PhosphorImager using the ImageQuant software (Molecular Dynamics).

GST Pull-down Assay—Approximately 5 μ g of purified GST fusion protein was incubated with 5 μ l of 35 S-labeled protein with moderate shaking at 4 $^{\circ}$ C overnight in binding buffer (20 mM HEPES, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl $_2$, 0.05% Nonidet P-40, 1 mM dithiothreitol, 1 mg/ml BSA). The bound protein was washed three times with binding buffer, and beads were collected by centrifugation. The

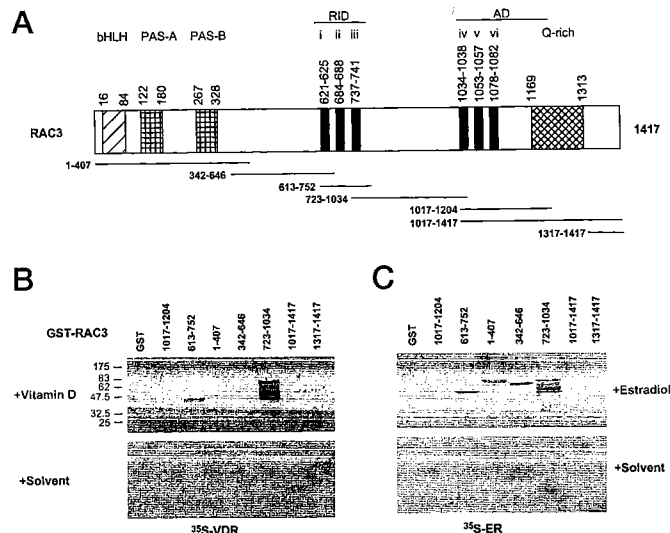


FIG. 1. The VDR and ER β interact with different fragments of RAC3. A, a schematic illustration of RAC3 and its functional domains as well as the purified GST-RAC3 fragments used for Far Western assays. AD, activation domain; i-vi, RAC3 LXXLL NR boxes. bHLH, basic helix-loop-helix. B, Far Western assay using 35 S-VDR to probe GST-RAC3 fusion proteins in the presence (top) or absence (bottom) of 1 μ M 1,25-dihydroxy-vitamin D $_3$. C, Far Western assay using 35 S-ER β to probe GST-RAC3 fusion proteins in the presence (top) or absence (bottom) of 1 μ M 17 β -estradiol.

bound protein was eluted in SDS sample buffer, subjected to SDS-polyacrylamide gel electrophoresis, and detected by autoradiography.

Site-directed Mutagenesis—NR box mutants were generated with the Quick-change site-directed mutagenesis system (Stratagene). The sequences of all mutant constructs were confirmed by dideoxynucleotide chain termination reactions using the T7 Sequenase protocol (U. S. Biochemical Corp.).

Gel Electrophoresis Mobility Shift Assay—The sequence of the DR3 element used for VDR/RXR gel-shift assays is AGCTTAAGAGGTCA-GAAAGGTCACTCGCAT. The double-stranded DR3 was end-labeled with [32 P]dCTP by standard Klenow fill-in reaction. The purified probe was incubated with 35 S-labeled receptors in binding buffer containing 7.5% glycerol, 20 mM HEPES, pH 7.5, 2 mM dithiothreitol, 0.1% Nonidet P-40, 1 μ g of poly(dI-dC) and 100 mM KCl. Wild-type or mutant GST-RAC3-RID was eluted from glutathione-agarose beads with 10 mM reduced glutathione and added to the binding reaction. The DNA-protein complex was formed on ice for 1 h and resolved on a 5% native polyacrylamide gel, which was subsequently dried and subjected to autoradiography.

Cell Culture and Transient Transfection—HEK293 and CV-1 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 5 μ g/ml gentamycin at 37 $^{\circ}$ C, 5% CO $_2$. Cells were plated for transfection in Dulbecco's modified Eagle's medium supplemented with 10% resin charcoal-stripped fetal bovine serum in 12- or 6-well plates 1 day before transfection. HEK293 cells were transfected using the standard calcium phosphate method, whereas CV-1 cells were transfected using LipofectAMINE according to the manufacturer's protocol (Life Technologies, Inc.). Twelve hours after transfection, cells were washed with phosphate-buffered saline and refed fresh medium containing the indicated concentration of ligand. After 24 h, cells were harvested for β -galactosidase and luciferase activities as described (35). Luciferase activity was determined with a MLX plate luminometer (Dynex) and normalized relative to β -galactosidase activity.

RESULTS

VDR and ER β Interact with Multiple Surfaces of RAC3—We have previously defined the minimal receptor interacting domain (RID) of RAC3 to be amino acids 613–752, which contains the first three LXXLL motifs (Fig. 1A) (20). We wished to further determine if different receptors were capable of binding to the same regions of RAC3. To accomplish this, we purified a panel of GST-RAC3 fusion proteins, in total comprising the full-length RAC3 (Fig. 1A), and probed these fusions with

[^{35}S]methionine-labeled VDR and ER β in a Far Western assay. The VDR, as expected, interacted in a ligand-dependent manner with GST-RAC3 613–752 in this assay (Fig. 1B). It also bound GST-RAC3 723–1034, which only contained NR box iii, in a ligand-dependent manner. The VDR did not interact with any other GST-RAC3 fragment, including GST-RAC3 342–646, which contained NR box i. It also appeared that the VDR interacted more strongly with GST-RAC3 723–1034 than with GST-RAC3 613–752, suggesting a more important role for NR box iii in the RAC3-VDR interaction. However, a different pattern was evident upon repeating this assay with ^{35}S -ER β , for in addition to ligand-dependent interactions with GST-RAC3 613–752 and 723–1034, ER β also bound the 342–646 fragment, which contained only NR box i (Fig. 1C). These interactions were of approximately equal intensity. There was also a weak, ligand-independent interaction with GST-RAC3 1–407. Identical results were obtained for ER α (data not shown). A Coomassie Blue-stained polyacrylamide gel of the GST-RAC3 fusion protein confirmed the identity of each GST-RAC3 fusion protein and approximately equal protein concentrations in each lane (data not shown). Thus, the VDR and ER β display different binding patterns for RAC3 fragments, with the VDR interacting preferentially with regions containing NR box iii and the ER β interacting equally well with regions containing any of the three NR boxes and the N-terminal basic helix-loop-helix-PAS domain.

NR Box Peptides Differentially Compete with Nuclear Receptors for RAC3 Binding—We then wanted to investigate the relative importance of individual NR boxes within the RAC3-RID in mediating the interactions between RAC3 and the VDR or ER β . Peptides were synthesized corresponding to NR boxes i, ii, and iii, which were incubated with ^{35}S -labeled receptor and 1 μM ligand before probing the GST-RAC3 613–752 fragment in the Far Western assay. With the VDR, peptides corresponding to the second and third LXXLL motif were able to compete away the RAC3-RID interaction with VDR in a dose-dependent manner (Fig. 2A). Upon quantifying the data, it was evident that peptide iii was a more potent inhibitor than peptide ii, whereas the peptide comprising NR box i had little, if any, effect on the VDR-RID interaction (Fig. 2B). A control experiment demonstrated that the effects of these peptides were specific, for a random peptide did not alter the interaction between the ^{35}S -VDR and GST-RAC3-RID (Fig. 2C). We again identified a different pattern when the same experiment was done with the ER β (Fig. 2D). Here, all three peptides were able to compete efficiently for ER β binding with the RAC3-RID, with peptide ii being the most potent (Fig. 2E). Thus, these data reveal receptor-specific preferences for interactions between RAC3 and different nuclear receptors, for the VDR and ER β have different affinities for the NR boxes of RAC3.

NR Box Mutations Can Impair RAC3 Interactions with Nuclear Receptors in Vitro—To assess the integrity of the LXXLL motif in mediating the interaction between the RAC3-RID and nuclear receptors, we used site-directed mutagenesis to switch the leucine residues of each motif to alanines (Fig. 3A). The mutants were made using the GST-RAC3-RID fusion as the template and tested for their ability to interact with the VDR or ER β in GST pull-down assays. The wild-type RAC3-RID was able to pull down a significant amount of ^{35}S -VDR in the presence of 1 μM vitamin D (Fig. 3B). This interaction was specific, for GST alone pulled down much less ^{35}S -VDR. Mutations in NR boxes i or ii displayed wild-type binding. However, when NR box iii was mutated, the RID-VDR interaction was greatly reduced to a level only slightly higher than background binding to GST alone. GST-RAC3 342–646, which contained only NR box i, also had minimal binding to the VDR, consistent

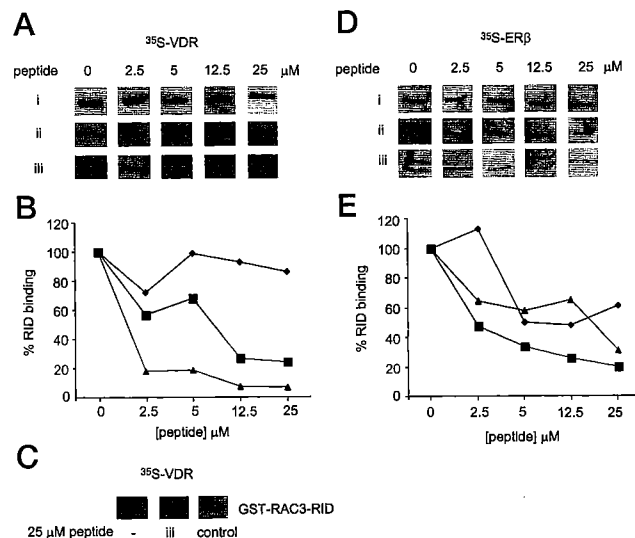


FIG. 2. Peptides corresponding to the NR boxes of the RAC3-RID can compete for VDR and ER β binding with the RAC3-RID.

A, Far Western assay using ^{35}S -VDR to probe GST-RAC3-RID in the presence of 1 μM vitamin D and given concentration of each peptide. B, the data from A was quantified by PhosphorImager and plotted as percent GST-RAC3-RID binding to ^{35}S -VDR versus peptide concentration. 100% RID binding represents the density of the band in the absence of peptide. \diamond , peptide i; \blacksquare , peptide ii; \blacktriangle , peptide iii. C, Far Western assay demonstrating that inhibition of ^{35}S -VDR interaction with GST-RAC3-RID by peptide iii is specific. Peptide iii abolishes nearly all the interaction, whereas a control, random peptide has no effect. D, Far Western assay using ^{35}S -ER β to probe GST-RAC3-RID in the presence of 1 μM estradiol and given concentration of each peptide. E, the data from D was quantified by PhosphorImager and plotted as the percent GST-RAC3-RID binding to ^{35}S -ER β versus peptide concentration. \diamond , peptide i; \blacksquare , peptide ii; \blacktriangle , peptide iii.

with the Far Western assay (Fig. 1B). Equal protein concentrations of each GST fusion confirmed the specificity of these findings. Thus, these data support the above observations in implicating NR box iii as being most critical to RAC3 interaction with the VDR.

The wild-type RID was also able to pull down significant amounts of ^{35}S -ER β in the GST pull-down assay (Fig. 3C). In contrast to the VDR, an alanine substitution for leucine in any of the three NR boxes weakened the interaction of the RAC3-RID with ER β , with the mutation of NR box ii being the most deleterious, again supporting the results of LXXLL peptide competition experiments (Fig. 2B). However, with each mutation, significant binding above background between the RAC3-RID and ^{35}S -ER β was still observed. Furthermore, the GST-RAC3 342–646 fragment, with only NR box i, was able to interact efficiently with ER β (Fig. 3C) as in the Far Western assay (Fig. 1C). These data suggest that although all three motifs are capable of interacting with ER β separately, none of them is absolutely required for the interaction. In contrast, NR box iii of RAC3 appears to be essential for the interaction with the VDR.

RAC3-RID Interactions with DNA-bound Nuclear Receptors—The above data provide compelling evidence that the VDR interacts specifically with RAC3 in solution via the LXXLL motifs of the RAC3 RID, particularly NR box iii. To gain further insight into the function of NR boxes in coactivator-VDR interactions on a heterodimeric complex bound to DNA, we performed gel-shift assays with VDR/RXR heterodimers on a DR3 element in the presence of wild-type or mutant RAC3-RID (Fig. 4). The VDR/RXR heterodimer bound strongly to the ^{32}P -labeled DR3 probe and was unaffected by the addition of GST alone (Fig. 4A, lanes 1 and 2). The addition of the RAC3-RID resulted in a ligand-dependent shift of the

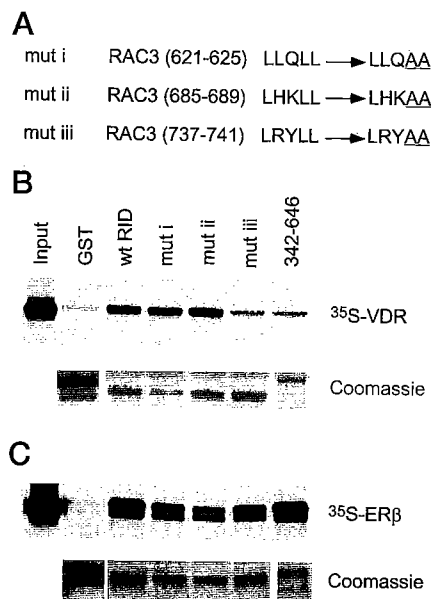


FIG. 3. Mutation of RAC3 NR boxes reveal different LXXLL motif preferences for VDR binding versus ERβ binding. **A**, alanine substitution for leucine in each of the RAC3-RID NR boxes by site-directed mutagenesis. *mut*, mutant. **B**, GST pull-down assay of ³⁵S-VDR and the indicated GST fusion proteins in the presence of 1 μM vitamin D. *wt*, wild type. The bottom panel confirms approximately equal GST-RAC3 RID protein concentrations in each lane by Coomassie Blue staining. **C**, GST pull-down assay of ³⁵S-ERβ and the indicated GST fusion proteins in the presence of 1 μM estradiol. The bottom panel confirms approximately equal protein concentrations in each lane by Coomassie Blue staining. The two observed bands are due to two ERβ products in the *in vitro* translation reaction.

heterodimeric complex to a slower-migrating form (arrow, lanes 3 and 4). The mutation of NR box i had little effect on the ability of the RID to shift the complex (lane 5). However, mutating NR box ii diminished the formation of the RID-VDR/RXR complex, whereas mutating NR box iii nearly abolished the formation completely (lanes 6 and 7). Consistently, GST-RAC3 342-646, with only NR box i, was unable to shift the VDR/RXR complex (lane 8). These results differ slightly from the GST pull down, in which only the NR box iii mutation inhibited interaction with VDR alone (Fig. 3B), suggesting that both motifs ii and iii may contribute to the interaction with DNA-bound VDR/RXR heterodimer.

We next analyzed the involvement of nuclear receptor AF-2 domains in regulating the interaction between the RAC3-RID and the VDR/RXR heterodimer (Fig. 4B). Using the gel-shift assay, we compared the ability of the RID to bind the wild-type heterodimer versus the VDR/RXR443 and VDR402/RXR heterodimers, in which the AF-2 domain of RXR or VDR had been deleted, respectively. As demonstrated above, the RID was able to bind the DNA-bound, wild-type VDR/RXR complex (Fig. 4B, lane 2). Deletion of the VDR AF-2 domain did not affect the formation of the heterodimer-DNA complex but resulted in the loss of the RID-shifted complex (lane 4). This suggests that the VDR AF-2 domain is required for interaction of RAC3 with the heterodimer and that RXR AF-2 domain alone is not sufficient for the interaction. Interestingly, deletion of the RXR AF-2 domain resulted in a much stronger shift of the heterodimeric complex by the RAC3-RID (lane 6) without affecting heterodimer formation (lane 5), suggesting that the RXR AF-2 domain can inhibit the interaction between RAC3 and VDR/RXR. The strong interaction was abolished upon deletion of the VDR AF-2 domain (lane 8), further supporting a requirement of VDR AF-2 helix 12 for RAC3 binding to the DNA-bound heterodimer.

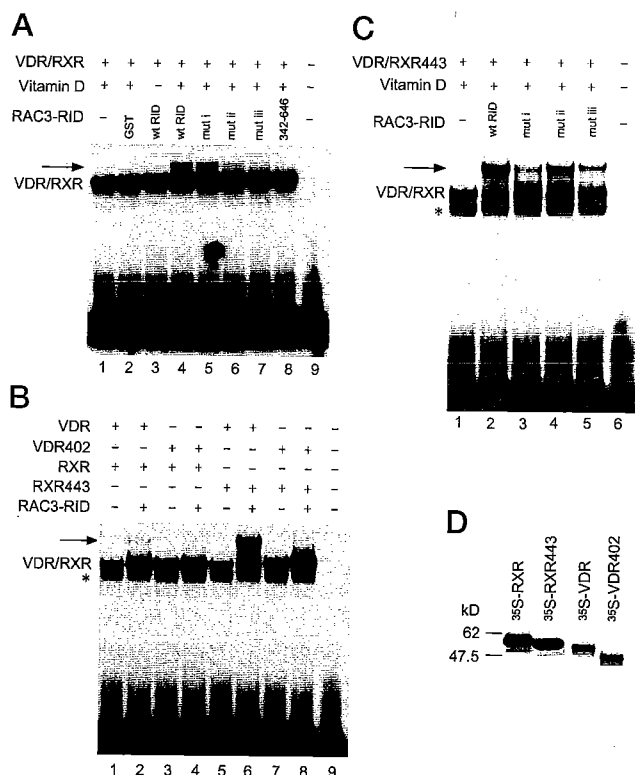


FIG. 4. Mutation of RAC3 NR boxes inhibits RAC3-RID binding to DNA-bound VDR/RXR heterodimer. **A**, gel-shift assay of the effects of NR box mutations (*mut*) on RAC3-RID binding to DR3-bound VDR/RXR. 1.5 μl of each ³⁵S-labeled nuclear receptor was added to a binding reaction (see "Experimental Procedures") containing 1 μM vitamin D, equal amounts of the indicated GST fusion protein, and the [³²P]dCTP-labeled DR3 probe. The arrow indicates the RID-receptor complex. *wt*, wild type. **B**, the RXR AF-2 domain can interfere with RID binding to VDR/RXR, whereas the VDR AF-2 domain is required for the interaction. The gel-shift assay was performed as in **A**, except the AF-2-truncated RXR443 or VDR402 was used where indicated. *, non-specific band from lysate. **C**, the VDR/RXR443 heterodimer has different NR box preferences than the wild-type receptor heterodimer. The gel-shift assay was performed as in **A**. *, non-specific band from lysate. **D**, autoradiograph confirming the equal expression of the ³⁵S-labeled receptors used in **B** and **C**.

Finally, we compared the RAC3 NR box preferences of VDR/RXR versus VDR/RXR443 (Fig. 4C). Intriguingly, the VDR/RXR443 heterodimer displayed different NR box preferences. Mutation of NR box i or iii greatly reduced the shift by the RAC3-RID (Fig. 4C, lanes 3 and 5), whereas mutation of NR box ii only slightly weakened the binding (lane 4). Thus deletion of the RXR AF-2 domain resulted in a switch in the NR box requirements, with NR boxes i and iii being most important for VDR/RXR443 compared with NR boxes ii and iii for VDR/RXR. An autoradiograph confirmed equal expression levels of each ³⁵S-labeled receptor (Fig. 4D). This finding supports the hypothesis that multiple LXXLL motifs provide RAC3 with the flexibility to adapt to different configurations of a nuclear receptor dimer.

Effects of NR Box Mutations on RAC3 Coactivation Function *in Vivo*—RAC3 has previously been shown to enhance the transcriptional activity of the RAR and progesterone receptor (37). However, its effect on VDR and ERβ function *in vivo* has not been demonstrated. To address this, we performed transient transfection assays in HEK293 and CV-1 cells using luciferase reporters harboring either two copies of the vitamin D response element of the osteopontin gene for VDR studies or a consensus ERE element for ERβ studies (Fig. 5). Transfection of the VDR into CV-1 cells minimally activated the vitamin D response element driven reporter (Fig. 5A). However, treating

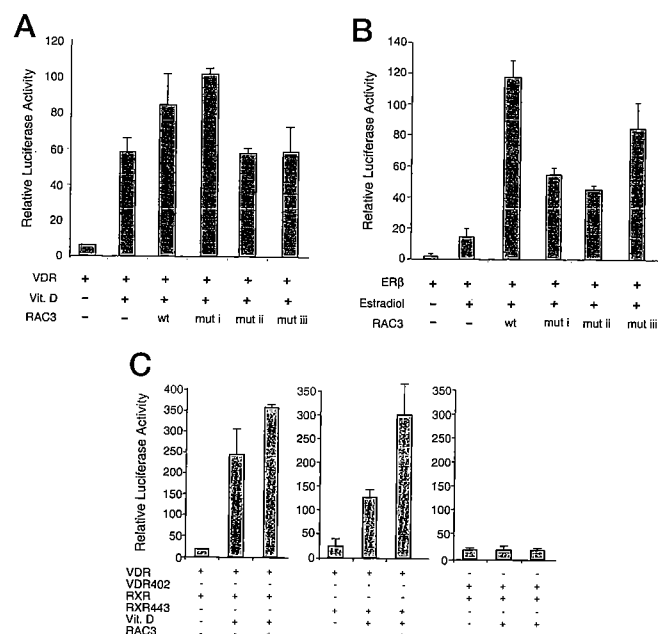


FIG. 5. Mutation of RAC3 NR boxes blocks coactivation of VDR and ER β activity *in vivo*. A, mutation (*mut*) of NR box ii or iii inhibits RAC3 enhancement of VDR activity. CV-1 cells were transfected with expression plasmids for VDR, wild-type (*wt*), or mutant RAC3 and the Sppx2-luciferase reporter and treated with 40 nM vitamin D for 24 h where indicated. Upon harvesting cells, luciferase activity was measured and normalized to β -galactosidase activity. B, mutation of NR box i, ii, or iii inhibits RAC3 enhancement of ER β activity. CV-1 cells were transfected with expression plasmids for ER β , wild-type, or mutant RAC3 and the ERE-luciferase reporter and treated with 10 nM estradiol for 24 h where indicated. Upon harvesting cells, luciferase activity was measured and normalized to β -galactosidase activity. C, deletion of the RXR AF-2 domain results in enhanced coactivation of VDR/RXR activity by RAC3. HEK293 cells were transfected with the appropriate nuclear receptor expression plasmids along with expression plasmids for RAC3 and Sppx2-luciferase and treated with 10 nM vitamin D where indicated. Upon harvesting cells, luciferase activity was measured and normalized to β -galactosidase activity.

these cells with vitamin D strongly stimulated its activity. Cotransfection of RAC3 further enhanced VDR transcriptional activation by approximately 50%, consistent with the coactivation function of RAC3 (37).

We then analyzed the role of the NR boxes in mediating the ability of RAC3 to potentiate VDR activity. Mutations of each NR box of the RAC3-RID were made in the context of the full-length RAC3 protein and tested for their ability to coactivate the VDR in transient transfection assays (Fig. 5A). Mutation of NR box i did not inhibit RAC3 enhancement of VDR transactivation, consistent with its inability to block the interaction of RAC3 with VDR *in vitro*. However, the RAC3- NR box ii or iii mutations reduced the function of RAC3 in enhancing VDR activity (Fig. 5A), consistent with their requirement for RAC3 binding to the DNA-bound VDR/RXR heterodimer in gel-shift assays (Fig. 4A). Taken together, it is clear that NR boxes ii and iii are both involved in RAC3 regulation of VDR function, whereas the role of NR box i appears minimal.

We then repeated these experiments with the ER β (Fig. 5B). Estradiol treatment of CV-1 cells transfected with the ER β and the ERE-luciferase reporter activated reporter expression approximately 8-fold. Cotransfection of wild-type RAC3 resulted in a strong enhancement of ER β activity. It is evident from these data that RAC3 is a more potent coactivator for the ER β than for the VDR. Cotransfection of RAC3 expression plasmids containing mutations in NR boxes i, ii, or iii all suppressed the ability of RAC3 to coactivate the ER β , with the NR box ii mutant having the greatest and NR box iii mutant having more

modest effects on RAC3 function. Thus, these *in vivo* data correlate with the *in vitro* data in implicating all three NR boxes of the RAC3-RID as being important for RAC3 regulation of ER β function.

Finally, we decided to assess the functional consequences of the antagonism of RAC3 interaction with DNA-bound VDR/RXR by the RXR AF-2 domain observed in gel-shift assays (Fig. 4B). To do this, we compared the ability of RAC3 to coactivate VDR/RXR and VDR/RXR443 activities by transient transfection assays (Fig. 5C). When wild-type VDR and RXR were expressed in HEK293 cells, RAC3 was able to enhance transcriptional activation by approximately 50% (Fig. 5C, left). However, when VDR was coexpressed with RXR443, RAC3 displayed a 2.5-fold enhancement of receptor activity (Fig. 5C, center). As expected, the VDR402 mutant was transcriptionally inactive, and RAC3 could not modulate its activity (Fig. 5C, right). Thus, these *in vivo* data are consistent with the gel-shift data in demonstrating that the RXR AF-2 domain can inhibit RAC3 modulation of the VDR/RXR heterodimer, whereas the VDR AF-2 domain is absolutely required for this regulation.

DISCUSSION

In this study, we have investigated the role of the NR boxes of RAC3 in mediating the ability of this coactivator to bind and coactivate the VDR and ER β . We found that NR box iii is most critical to VDR binding, whereas NR boxes i, ii, and iii are involved in ER β interaction. Peptides corresponding to these respective motifs were able to compete with the RAC3-RID for VDR and ER β binding. The integrity of the motifs themselves was also important, for mutations in specific NR boxes inhibited RAC3 interaction with these receptors in solution and when bound to DNA. The AF-2 domain of VDR is required for binding of the RAC3-RID to DNA-bound VDR/RXR, whereas the AF-2 domain of RXR was able to antagonize this interaction. Removal of this inhibitory AF-2 helix of RXR enhances ligand-dependent binding of RAC3 to the VDR/RXR443 heterodimer and alters the NR box requirements. Furthermore, the mutation of NR box ii or iii blocked the ability of RAC3 to enhance transcriptional activation by the VDR *in vivo*. In contrast, mutation of NR boxes i, ii, or iii reduced RAC3 coactivation of ER β . Together, these *in vitro* and *in vivo* studies suggest a mechanistic difference in the manner by which RAC3 regulates VDR and ER β activities.

The NR boxes are highly conserved among the SRC family of coactivators (18). Our data and that of others clearly reveal that multiple motifs are necessary for high affinity interactions with nuclear receptors (23, 32, 33). With the DNA-bound VDR/RXR heterodimer, we found that mutation of NR boxes ii or iii of RAC3 weakens the interaction with the RAC3-RID. In contrast, NR box iii of RAC3 is the only critical motif for interaction with VDR in solution. Therefore, it is likely that each motif binds to each monomer of the receptor heterodimer, consistent with the structure of a peroxisome proliferator-activated receptor γ -LBD dimer co-crystallized with a fragment of SRC1 containing two NR boxes (12). Our data on ER β suggest that all three NR boxes of the RAC3-RID are involved for a wild-type interaction, whereas the presence of two motifs is sufficient for a strong interaction, motif ii being most important. In light of this finding, RAC3 may utilize motif ii in combination with motif i or iii for efficient interaction with the ER β homodimer. The integrity of the other motif may be critical to the overall conformation of the coactivator or potentially make an additional contact with another region of the receptor. Support for the latter possibility can be found in recent studies detailing the enhancement of the N-terminal AF-1 activation function of nuclear receptors by SRC coactivators (38, 39). The presence of multiple NR boxes also likely provides coactivators the flexi-

bility to interact with a broad range of nuclear receptors, resulting in the different preferences that are observed between nuclear receptors and distinct motifs, depending on the precise structural nuances of each receptor-coactivator interface. This is evident upon comparing the NR box requirements of the VDR/RXR heterodimer *versus* those of VDR/RXR443. Deletion of the AF-2 helix of RXR not only enhances RAC3-RID binding to the heterodimer but also switches the NR box preferences from motifs ii/iii to motifs i/iii. Finally, amino acids flanking the NR boxes also likely contribute to the specificity of interaction (15, 32), for, despite the high homology between the RAC3 NR boxes, peptides comprising each motif and surrounding residues displayed different affinities for VDR or ER β binding. Thus, it is clear that the multiple NR boxes do not serve merely redundant functions.

Our finding that the AF-2 domain of RXR can interfere with RAC3-RID binding to a DNA-bound VDR/RXR heterodimer is consistent with studies suggesting allosteric inhibition of coactivator binding to RAR/RXR by the RXR AF-2 domain (36). This inhibition may be the result of competition between the AF-2 domain of RXR and the LXXLL motif for the coactivator binding site on the other receptor (11, 12, 36). In the antagonist-bound ER α -LBD crystal structure, the AF-2 domain occupies the coactivator binding groove, mimicking the hydrophobic interactions of the NR box peptide with this domain in the agonist-NR box peptide-receptor complex (11). Biochemical studies with RAR/RXR and SRC1 support these observations, for binding of RAR- and RXR-specific ligands enhance SRC1 interaction with the receptor dimer relative to the interaction in the presence of either ligand alone (36). Presumably, one ligand binding recruits a single NR box to the receptor dimer, which displaces the AF-2 domain from the coactivator binding site and relieves allosteric inhibition, allowing the second ligand to bind the other receptor monomer. This, in turn, enhances the interaction with coactivator by recruiting a second NR box (36). In the case of wild-type receptors, hormone does stimulate RAC3-RID binding to the heterodimer, but only weakly compared with the VDR/RXR443 dimer, where a very strong, vitamin D-dependent interaction is observed. These observations are confirmed by *in vivo* studies demonstrating that RAC3 can coactivate VDR/RXR443 activity to a greater extent than VDR/RXR activity. Hormone binding and RID recruitment must not be able to displace every RXR AF-2 domain from the coactivator binding site of the partnering receptor; thus, fewer RID molecules are able to bind in the presence of the RXR AF-2 domain. This suggests that the AF-2 domain of RXR plays a critical role in regulating RAC3 modulation of receptor function. However, other possibilities may explain this finding, foremost being the hypothesis that deletion of the AF-2 domain of RXR results in a conformational change of the VDR/RXR443 dimer that enhances its affinity for the RAC3-RID.

Our data demonstrate for the first time that RAC3 can enhance the transcriptional activation function of the VDR and ER β and that this coactivation activity depends on different NR box requirements. Several other cofactors have been found to stimulate VDR activity, including SRC1, GRIP1/TIF2, NCoA-62, and the DRIP (VDR-interacting proteins) complex (13, 40–42), whereas SRC1 can coactivate the ER β (43). The role of multiple coactivators in the function of the VDR *in vivo* is unknown, but several possibilities exist that suggest that the function of these coactivators is not completely redundant. First, the relative contribution of each coactivator may depend on cell or tissue type and/or coactivator levels in these cells. RAC3 is expressed at a high level in placenta, heart, and HeLa cells relative to TIF2 and SRC1 (20); thus, it may serve a more prominent role in receptor function in these cells. Second, dif-

ferent coactivators may serve different functions that in total result in maximal transcriptional activation by the VDR. For example, RAC3 can interact with CBP; thus, RAC3 may recruit CBP to the VDR. SRC1 has intrinsic histone acetylation activity, and the DRIP VDR-interacting proteins complex may remodel nucleosomes (21, 44), which also may contribute to the overall function of the VDR in stimulating target gene expression. Finally, we cannot rule out the existence of a complex containing multiple coactivators, which synergize to potentiate VDR activity.

In summary, our data establish RAC3 as a potent coactivator of the vitamin D receptor and estrogen receptor β . Interestingly, RAC3 modulates the function of these receptors differently via interactions that depend on specific LXXLL motifs in the RAC3 receptor-interacting domain. Although the biological role of RAC3 in nuclear receptor function remains to be explored, this study sheds light on the molecular mechanisms of RAC3 regulation of receptors that will hopefully lead to a better understanding of SRC coactivator function *in vivo*.

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Review

The SRC family of nuclear receptor coactivators

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Abstract

Nuclear hormone receptors are ligand-dependent transcription factors that regulate genes critical to such biological processes as development, reproduction, and homeostasis. Interestingly, these receptors can function as molecular switches, alternating between states of transcriptional repression and activation, depending on the absence or presence of cognate hormone, respectively. In the absence of hormone, several nuclear receptors actively repress transcription of target genes via interactions with the nuclear receptor corepressors SMRT and NCoR. Upon binding of hormone, these corepressors dissociate away from the DNA-bound receptor, which subsequently recruits a nuclear receptor coactivator (NCoA) complex. Prominent among these coactivators is the SRC (steroid receptor coactivator) family, which consists of SRC-1, TIF2/GRIP1, and RAC3/ACTR/pCIP/AIB-1. These cofactors interact with nuclear receptors in a ligand-dependent manner and enhance transcriptional activation by the receptor via histone acetylation/methylation and recruitment of additional cofactors such as CBP/p300. This review focuses on the mechanism of action of SRC coactivators in terms of interactions with receptors and activation of transcription. Specifically, the roles of the highly conserved LXXLL motifs in mediating SRC function will be detailed. Additionally, potential diversity among SRC family members, as well as several recently cloned SRC-associated cofactors, will be discussed. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: CBP; LXXLL motif; RAC3/ACTR/pCIP/AIB-1; Steroid receptor coactivator; TIF2/GRIP1

1. Introduction

The steroid and thyroid nuclear receptor superfamily is a large class of ligand-dependent transcription factors involved in the regulation of genes that play critical roles in a wide array of biological processes, including development, reproduction, and homeostasis (Mangelsdorf et al., 1995). This superfamily can be

further subdivided into three classes of nuclear receptors. Type I or steroid receptors include those for estrogens (ER), progestins (PR), androgens (AR), glucocorticoids (GR), and mineralcorticoids (MR). Type I receptors are coupled to heat-shock proteins and sequestered to the cytoplasm in the absence of ligand (Tsai and O'Malley, 1994). Upon hormone binding, they dissociate from the heat-shock proteins, homodimerize, and translocate to the nucleus, where they bind cognate response elements consisting of palindromic repeats. Type II receptors include those for all-*trans* retinoic acid (RAR), thyroid hormone (TR), and vitamin D (VDR). These receptors are strictly nuclear and form heterodimers with the receptor for 9-*cis* retinoic acid (RXR). They also bind constitutively to response elements consisting of direct repeats. A third class of nuclear receptors is the orphan receptors, so-called because endogenous ligands for these proteins are currently unknown.

Most members of the nuclear receptor superfamily share a common domain structure. The N-terminus

Abbreviations: ACTR, activator of retinoic acid receptor; AF-2, activation function-2 domain; AIB-1, amplified in breast cancer-1; CARM1, coactivator-associated arginine methyltransferase; GRIP1, glucocorticoid receptor-interacting protein 1; HAT, histone acetyltransferase; LBD, ligand binding domain; NR box, nuclear receptor interacting box; pCIP, p300/CBP cointegrator-associated protein; RAC3, receptor associated coactivator 3; SRA, steroid receptor RNA activator; SRC, steroid receptor coactivator; TIF2, transcription intermediary factor 2; TRAM-1, thyroid receptor activator molecule 1.

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contains the variable A/B region, which also includes the ligand-independent activation function-1 (AF-1) domain. The C region represents the highly conserved DNA-binding domain (DBD) and is followed by the hinge region (D) and the C-terminal ligand-binding domain (LBD) (E). The LBD contains the ligand-dependent activation function-2 (AF-2) domain and also mediates dimerization of nuclear receptors. In the absence of ligand, several nuclear receptors are able to repress basal transcription via recruitment of the nuclear receptor corepressors SMRT and NCoR (Horlein et al., 1995; Chen and Evans, 1995). SMRT and NCoR are found in complexes with the corepressor mSin3 and histone deacetylases (HDACs), suggesting that transcriptional repression by nuclear receptors may involve histone deacetylation (Nagy et al., 1997; Heinzel et al., 1997; Alland et al., 1997). Ligand binding triggers the release of these corepressors and subsequent recruitment of coactivators through a drastic conformational change in the AF-2 domain of the receptor. Structural studies have demonstrated that helix 12 within the AF-2 domain projects away from the LBD in the unliganded structure (Renaud et al., 1995; Wagner et al., 1995; Bourguet et al., 1995; Brzozowski et al., 1997). This helix rotates nearly 180° to pack tightly against the LBD upon hormone binding in the RAR, TR, and ER crystal structures. This conformational change, together with ligand-induced changes in helices 3–5, is believed to facilitate interactions of the receptor with coactivators (Shiau et al., 1998; Darimont et al., 1998; Feng et al., 1998; Nolte et al., 1998). Subsequently, coactivators are able to enhance transcriptional activation by the receptor via mechanisms that include recruitment of the general coactivator CBP/p300 and histone acetylation.

Coactivators are generally defined as proteins that can interact with DNA-bound nuclear receptors and enhance their transcriptional activation function. Although many nuclear receptor coactivators have been identified (McKenna et al., 1999), the steroid receptor coactivator (SRC) family has been the focus of intense study in recent years. Thus this review will focus on the mechanisms of action of these cofactors in regulating the function of nuclear receptors and also highlight several of the recently cloned SRC-associated proteins.

2. The SRC family of coactivators

The first nuclear receptor coactivator, steroid receptor coactivator-1 (SRC-1), was cloned by using the PR-LBD as bait in a yeast-two-hybrid screen of a human B-cell cDNA library (Oñate et al., 1995). SRC-1 interacts in a ligand-dependent manner with and enhances AF-2 transcriptional activation by a broad range of nuclear receptors, including PR, ER, TR, RXR, GR, and PPAR. Recent data also detail the enhancement of ER (Webb

et al., 1998) and AR (Alen et al., 1999; Bevan et al., 1999; Ma et al., 1999) AF-1 activities by SRC-1. In addition, SRC-1 has been demonstrated to interact with the general transcription factors TBP and TFIIB, although the functional consequences of these interactions are unknown (Takeshita et al., 1996; Ikeda et al., 1999). Furthermore, SRC-1 is able to enhance transcriptional activation mediated by NF- κ B, SMAD3, and AP-1 (Lee et al., 1998; Yanagisawa et al., 1999; Na et al., 1998), supporting a role for nuclear receptor coactivators in multiple intracellular signaling pathways. Subsequent studies have identified two functionally distinct SRC-1 isoforms, SRC-1a and SRC-1e, which contain unique C-termini, suggesting that alternative splicing may regulate SRC-1 function (Kalkhoven et al., 1998).

The identification of transcription intermediary factor 2 (TIF2) and GR-interacting protein 1 (GRIP1) established the SRC family of coactivators (Voegel et al., 1996; Hong et al., 1996). TIF2 was isolated in a Far-western screen as an ER- and RAR-interacting factor, while GRIP1 was isolated using the GR-LBD as bait in a yeast-two-hybrid screen. TIF2 and GRIP1 share 94% amino acid identity, thus represent the human and murine orthologs, respectively. TIF2 and GRIP1 associate in vivo with hormone-bound RAR, ER, and PR and coactivate ligand-dependent transactivation. Like SRC-1, GRIP1 also has been demonstrated to enhance receptor AF-1 activity in addition to that of the AF-2 domain (Ma et al., 1999). Intriguingly, the inv(8)(p11q13) chromosomal translocation results in a fusion between TIF2 and the MOZ gene, which contributes to the pathogenesis of acute myeloid leukemia (AML), suggesting a role for transcriptional regulation by nuclear receptor coactivators in these leukemias (Carapeti et al., 1998).

The third member of the SRC family was reported simultaneously by several groups as an RAR-interacting protein (RAC3), a CBP-interacting protein (p/CIP), a hRAR β -stimulatory protein (ACTR), a gene amplified in breast cancer (AIB-1), and a TR-interacting protein (TRAM-1) (Li et al., 1997; Torchia et al., 1997; Chen et al., 1997; Anzick et al., 1997; Takeshita et al., 1997). p/CIP represents the mouse homolog, while RAC3/ACTR/AIB-1/TRAM are human isoforms. In addition to coactivating many nuclear receptors, pCIP has also been demonstrated to enhance the activity of interferon- α and cAMP regulatory element binding protein (CREB), suggesting that this coactivator may be involved in multiple signaling pathways (Torchia et al., 1997). Furthermore, RAC3/TRAM-1 expression can be upregulated by hormone treatment, which represents another possible mechanism by which coactivators may potentiate hormone action (Li and Chen, 1998; Misiti et al., 1998).

3. The LXXLL motif

The SRC family of coactivators also shares a common domain structure, with the most highly conserved region being the N-terminal bHLH–PAS domain (Fig. 1a). The bHLH region functions as a DNA-binding or dimerization surface in many transcription factors, including the MyoD family of proteins (Murre et al., 1989a, b). The PAS motif is also found in several transcriptional regulators, including Period (Per), Aryl hydrocarbon receptor (AhR), and single-minded (Sim). Similar to the bHLH domain, the PAS domain also plays a role in protein–protein interactions and dimerization. However, the function of the bHLH–PAS domains of SRC coactivators remains unknown, though it is likely to mediate intra- or intermolecular interactions. This bHLH–PAS

domain is followed by a centrally located receptor-interacting domain (RID) and C-terminal transcriptional activation domain (AD), which will be discussed in detail below.

The RID of SRC coactivators mediates ligand-dependent, direct interactions with nuclear receptors (Li and Chen, 1998; Voegel et al., 1998; Oñate et al., 1998). Intriguingly, detailed analysis of the sequence of the RID identified a conserved motif, LXXLL, where L is leucine and X is any amino acid, that is termed the NR box (Heery et al., 1997) (Fig. 1b). Three such motifs are found in the RID of SRC coactivators, with an additional, non-conserved NR box also present at the C-terminus of the SRC-1 isoform SRC-1a. Site-directed mutagenesis and peptide competition experiments have provided strong evidence for the requirement of these

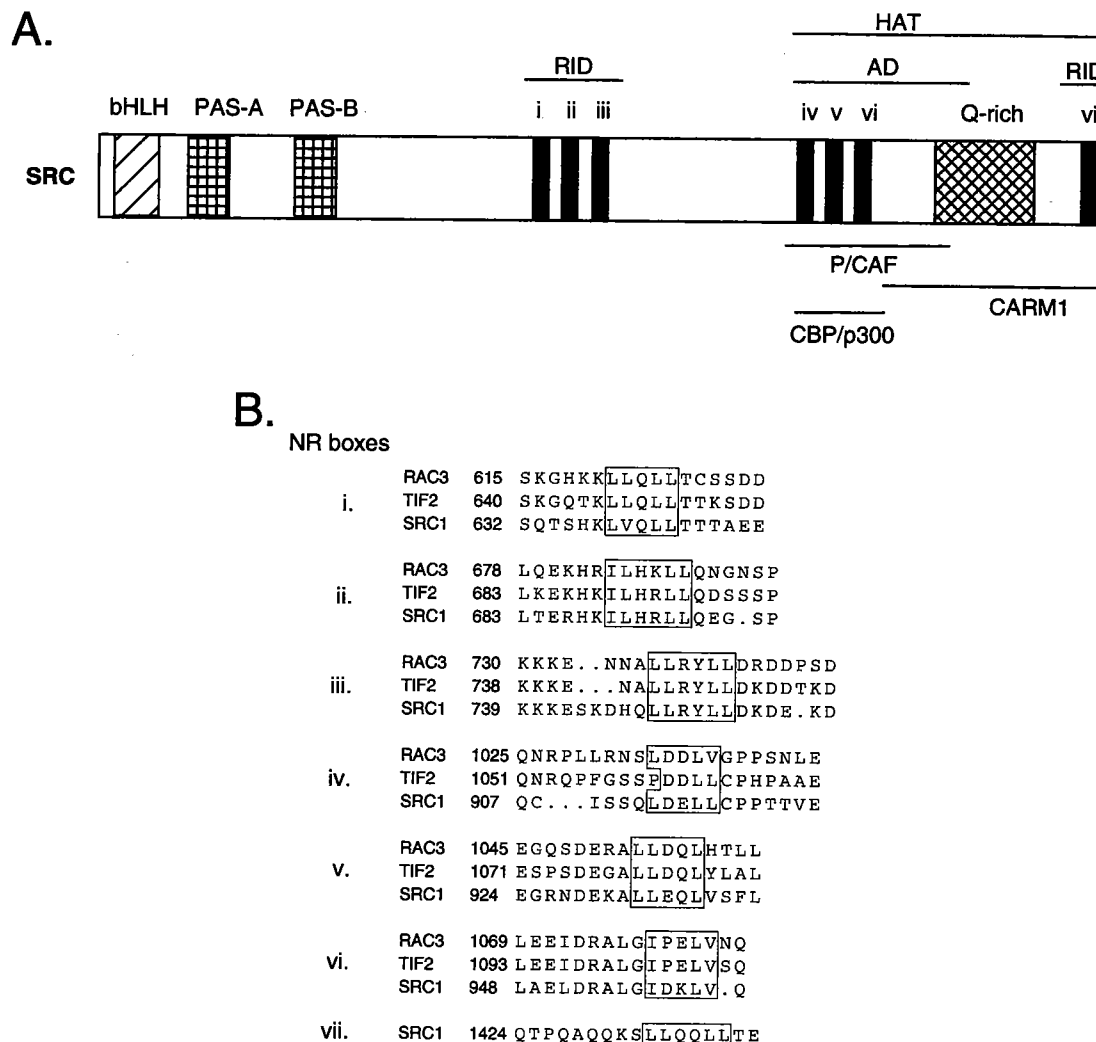


Fig. 1. SRC family domain structure. (A) Schematic representation of the structural domains of SRC coactivators. The N-terminus contains the highly conserved bHLH and PAS A/B domains. The centrally located receptor-interacting (RID) and activation (AD) domains each contain three LXXLL motifs, while SRC-1 contains an additional, non-conserved motif at the C-terminus. The specific domains for interaction with P/CAF, CBP/p300, and CARM1, as well as the histone acetyltransferase (HAT) domain, are indicated. (B) Sequence alignment of the SRC LXXLL motifs. The starting amino acids are in parentheses. Motifs i–iii are located in the receptor-interacting domain and motifs iv–vi are found in the transcriptional activation domain. SRC-1 contains an additional non-conserved motif at its C-terminus.

motifs for mediating interactions between coactivators and liganded nuclear receptors (Heery et al., 1997; Torchia et al., 1997; Ding et al., 1998). Further support for the role of these motifs in mediating agonist-dependent interactions with nuclear receptors is found in a study in which phage-displayed peptide libraries were screened for peptides that interact specifically with agonist or antagonist bound estrogen receptor (Norris et al., 1999). Many peptides isolated with estradiol-bound ER α contained the LXXLL motif, while those isolated with tamoxifen-bound receptor did not. These findings suggest that the activation of the ER α by tamoxifen that is observed in some tissues might occur via a different mechanism than estradiol-induced activation, such as through the recruitment of non-LXXLL containing coactivators to tamoxifen-specific surfaces of the ER. Crystallographic and protein structure prediction analyses have indicated that these motifs form amphipathic α -helices with the leucine residues comprising a hydrophobic surface on one face of the helix. The helix is able to interact with the AF-2 domain of the liganded receptor via a hydrophobic groove made up of residues from receptor helices 3, 4, 5, and 12 that is the result of the conformational change induced by hormone binding (Torchia et al., 1997; Shiau et al., 1998; Nolte et al., 1998; Darimont et al., 1998; Feng et al., 1998).

The most interesting aspect of NR box function is the revelation that a receptor-specific code exists, where different nuclear receptors prefer different NR boxes of the RID for interaction with coactivators (Leers et al., 1998; Ding et al., 1998; Darimont et al., 1998; McInerney et al., 1998). For example, a 13-aa peptide encompassing GRIP1 motif ii efficiently blocked interaction between GRIP1 and the TR β -LBD in vitro, while a peptide comprising motif iii was a more potent competitor for GR binding (Darimont et al., 1998). Similarly, yeast-two-hybrid assays demonstrate that mutation of TIF2 motif ii is most deleterious to interactions with PPAR α , while a motif i mutation has the greatest effect on the TIF2-RXR β interaction (Leers et al., 1998). In all cases, however, mutation of a single motif does not completely abolish coactivator interaction with nuclear receptors, suggesting that multiple NR boxes contribute to the overall, high-affinity binding to the receptor. It is likely that the precise arrangement of multiple motifs and structural nuances of each receptor determines the relative contribution of each NR box to the interaction.

This receptor-specific code has also been analyzed in vivo in terms of transcriptional coactivation of nuclear receptors by SRC-1 via site-directed mutagenesis and antibody microinjection assays (McInerney et al., 1998). The requirement of specific NR boxes for transactivation of reporter genes by different receptors was determined by injecting anti-SRC-1 antibodies into cells along with rescuing plasmids for wild-type or NR box mutants of SRC-1. Anti-SRC-1 IgG completely abolishes transcrip-

tional activation by ER, PR, RAR, TR, and PPAR γ , while coinjection of wild-type SRC-1 rescues receptor function. Mutation of NR box ii prevented rescue of ER function in SRC-1 immunodepleted cells, while NR boxes ii and iii were required for rescue of RAR and TR activity and boxes i and ii for PR activity. Furthermore, in the case of PPAR γ , different ligands elicited different NR box requirements for SRC-1 coactivation. Troglitazone-bound PPAR γ preferred NR box ii over box i, while the opposite was observed in indomethacin-treated cells. Together, these data support receptor-specific LXXLL motif requirements for coactivation function and receptor interactions that account for the presence of multiple NR boxes within SRC coactivators and imply that these motifs do not serve merely redundant functions.

Other determinants that contribute to the specificity of NR box selectivity by different nuclear receptors include residues flanking each NR box. For instance, a chimeric peptide containing the GRIP1 NR box iii motif in the context of the flanking sequences of NR box ii competed for TR-LBD binding with a similar potency as the peptide comprising NR box ii (Darimont et al., 1998). Also, using phage-displayed libraries enriched for LXXLL-containing peptides, it was demonstrated that several subclasses of these peptides exist which contain different flanking residues and which vary in their abilities to interact with different ER mutants and other receptors (Chang et al., 1999). Furthermore, it has been shown that the flanking N-terminal amino acids are not essential, while the eight residues C-terminal to the NR box are required for SRC-1 mediated coactivation of RAR, TR, and ER (McInerney et al., 1998). These studies also revealed additional preferences of ER and RAR for different NR box ii C-terminal amino acids. Intact residues +12 and +13 (where L of LXXLL is +1) are required for SRC-1 rescue of ER activity, while residues at +6, +7, +11, and +13 are necessary for rescue of RAR function. Finally, since most nuclear receptors require two intact NR boxes of coactivator for interaction, the spacing between the motifs also can serve as a determinant for recognition. Deletion of 30 of the 50 amino acids between NR boxes ii and iii abolished the ability of SRC-1 to rescue IgG-mediated inhibition of RAR activity (McInerney et al., 1998). In contrast, proper spacing between NR boxes i and ii was required for coactivation of PPAR γ , consistent with the requirement of intact motifs i and ii for maximal PPAR γ transactivation.

4. X-Ray crystal structures

The biochemical studies outlined above clearly outline the LXXLL motifs of SRC coactivators as being critical to the interaction with and coactivation of nuclear

receptors. Further insight into the molecular basis of these interactions can be found in the recently solved crystal structures of several nuclear receptor LBDs with coactivator fragments containing NR boxes. In the structure of TR β -LBD complexed with T3 and a 13-aa peptide encompassing NR box ii of GRIP1, the leucines of the α -helical NR box make contacts with a hydrophobic groove consisting of residues from helices 3, 4, 5, and 12 of TR β (Darimont et al., 1998). A single LXXLL peptide interacts with each monomer of the TR β dimer. Mutagenesis confirms the importance of these receptor residues for *in vitro* binding of GRIP1 to TR β . A very similar structure is observed with agonist-bound ER α -LBD complexed with a peptide comprising NR box ii of GRIP1 (Shiau et al., 1998). However, in the antagonist-bound ER α -LBD structure, helix 12 of the ER α is occluding the coactivator-binding site, consistent with the inability of SRC coactivators to bind antagonist-bound nuclear receptors. Most strikingly, a region of helix 12 contains an NR box-like sequence (LXXML) and functions as an intramolecular mimic of the LXXLL motif by making contact with the hydrophobic groove. This structural data supports the model of allosteric inhibition of the RXR–RAR heterodimer by the RXR AF-2 domain (Westin et al., 1998). These observations provide a molecular basis of antagonist function via conformational changes of helix 12 and inhibition of coactivator binding. Finally, the structure of the PPAR γ -LBD bound to rosiglitazone and an 88-aa fragment of SRC-1 containing NR boxes i and ii has also been described (Nolte et al., 1998). This study details the function of a “charge clamp” of conserved glutamine and lysine residues of the LBD that positions the LXXLL motif into the hydrophobic groove of the receptor. In addition, the two NR boxes of the SRC-1 fragment are observed to contact simultaneously the PPAR γ dimer, providing further support for the role of multiple motifs in mediating coactivator–receptor interactions.

5. Transcriptional activation by SRC coactivators

The SRC coactivators also contain an intrinsic transcriptional activation function, which is evident upon tethering coactivator to DNA via a heterologous DNA-binding domain. All three members are able to efficiently activate transcription when fused to the Gal4 DNA-binding domain in both yeast and mammalian cells (Li et al., 1997; Oñate et al., 1998; Voegel et al., 1996). Detailed deletion analysis has subsequently mapped the activation domain (AD) as being located C-terminal to the receptor-interacting domain. Interestingly, this AD also contains three additional LXXLL motifs representing NR boxes iv, v, and vi, that have been linked to interaction with the general transcriptional activators CBP/p300. CBP/p300 has been demonstrated to interact

with SRC proteins *in vitro* and *in vivo* and mutation of one or more of the AD NR boxes markedly impairs these interactions, as well as the activation function of the coactivator (Voegel et al., 1998; McInerney et al., 1998). Furthermore, microinjection studies have shown that anti-CBP antibodies abolish the ability of SRC-1 to coactivate RAR, suggesting that CBP/p300 is required for the coactivation function of SRC-1 (McInerney et al., 1998). However, it is worth noting that additional, CBP-independent transcriptional activation domains have also been attributed to members of the SRC family, supporting the existence of multiple mechanisms of transcriptional activation by coactivators (Ikeda et al., 1999; Ma et al., 1999; Voegel et al., 1998). The contribution of these multiple activation domains to overall coactivator function is not completely clear, but several studies suggest that coactivators may preferentially utilize specific ADs depending on the receptor or activation function (AF-1 vs. AF-2) that is mediating the response to hormone (Ma et al., 1999). In particular, the N-terminal AF-1 activation domain seems to be most critical to transcriptional activation by the androgen receptor (AR). SRC-1 and GRIP1 have been demonstrated to bind and coactivate the AF-1 domain of the AR (Alen et al., 1999; Bevan et al., 1999; Ma et al., 1999). These interactions are mediated by the C-terminus of the SRC coactivator, rather than the centrally located domain containing NR boxes i/ii/iii. Consistent with this observation, mutation of these motifs does not inhibit the ability of the coactivator to enhance transcriptional activation by the full-length AR, but only the isolated AF-2 domain (Alen et al., 1999; Bevan et al., 1999). This AF-1 interacting domain also lacks the CBP-interacting domain of the coactivator, thus coactivation of AF-1 likely occurs via mechanisms other than CBP recruitment, but through additional coactivators, such as CARM1 (see below), which also binds the C-terminus of GRIP1 (Chen et al., 1999a). Interestingly, the N-terminus of the AR also can interact with the C-terminal AF-2 domain and is required for both AF-2-induced transcription and SRC coactivation of AF-2 activity (Alen et al., 1999). These studies suggest that the AF-1 and AF-2 domains may synergize for complete AR activity and that the interaction of the two activation domains may result in recruitment of coactivator to the AR. Finally, SRC coactivators have also been demonstrated to enhance the AF-1 activity of the ER α in the presence of both estradiol and tamoxifen, suggesting that the partial agonism of tamoxifen occurs by coactivator recruitment to the AF-1 domain (Webb et al., 1998).

Another potential mechanism of transcriptional activation by SRC coactivators is histone acetylation. Hyperacetylated histones have long been linked to transcriptionally active chromatin, for acetylation leads to an unpacking of the condensed chromatin network, thereby facilitating the access of transcription factors to

target gene promoters. Accordingly, both CBP/p300 and the CBP/p300-associated factor P/CAF have been demonstrated to possess potent histone acetyltransferase (HAT) activity (Bannister and Kouzarides, 1996; Ogryzko et al., 1996; Yang et al., 1996), which is required for transcriptional activation by CBP (Martinez-Balbas et al., 1998). Both factors interact with SRC coactivators, as well as with nuclear receptors themselves, and enhance receptor transcriptional activation (Kamei et al., 1996; Blanco et al., 1998; Chakravarti et al., 1996). Therefore, recruitment of HAT-containing coactivators by the receptor may lead to a modulation of chromatin structure, thereby facilitating the access of either additional transcriptional activators, such as the DRIP/TRAP complex (see below), or the assembly of the pre-initiation complex, ultimately leading to transcriptional activation. Interactions between coactivators and the basal transcription machinery may also play a role in transcriptional activation, for both CBP/p300 (Kwok et al., 1994; Yuan et al., 1996) and SRC-1 (Ikeda et al., 1999; Takeshita et al., 1996) have been reported to interact with TBP and TFIIB. Interestingly, moderate HAT activity has also been attributed to SRC-1 and ACTR, suggesting that liganded nuclear receptors recruit a coactivator complex containing multiple enzymatic activities (Chen et al., 1997; Spencer et al., 1997). The apparent redundancy of HAT activities among the coactivator complex remains to be resolved completely. However, transcription factor-specific differences in HAT requirements have been established for RAR versus CREB via microinjection analysis (Korzus et al., 1998). Whereas P/CAF HAT activity was required for transcriptional activation by RAR, CBP HAT activity was required for CREB function. Additionally, cell-type and promoter-specific differences may also account for the existence of numerous HAT-containing coactivators. Finally, multiple HAT activities may be required if non-histone proteins also serve as substrates for these enzymes. In support of this, p300 has been demonstrated to acetylate p53, increasing its DNA binding activity (Gu and Roeder, 1997). Also, CBP and P/CAF can acetylate TFIIE and TFIIIF *in vitro*, which again links the basal transcription machinery to transcriptional activation by nuclear receptor coactivators (Imhof et al., 1997). Furthermore, a recent study reports that ACTR itself can be acetylated by CBP/p300 (Chen et al., 1999b). In this paper, hormone treatment results in enhanced histone acetylation at ER, RAR, and VDR target gene promoters and increased recruitment of coactivators, including ACTR and CBP/p300. However, this effect is transient in nature and is strongly downregulated after prolonged hormone treatment. Surprisingly, acetylation of ACTR by CBP/p300 at specific lysine residues causes the dissociation of ACTR from the DNA-bound ER homodimer. These results suggest that the mechanism of downregula-

tion of receptor activity involves release of the coactivator complex from the receptor via acetylation of the coactivator itself.

6. Diversity among SRC coactivators

One of the most important remaining questions to be answered concerning the function of SRC coactivators *in vivo* focuses on whether or not these three cofactors serve redundant functions. Although all three SRC family members do possess similar properties in terms of interactions with nuclear receptors and enhancement of transcriptional activation, several reports suggest that their activities are not completely overlapping and particularly outline a division between SRC-1 and TIF2/GRIP1 versus RAC3/ACTR/pCIP/AIB-1 functions. For example, microinjection of expression plasmids for SRC-1 or NCoA-2, but not pCIP, were able to rescue RAR-dependent activation in SRC-1 immunodepleted cells (Torchia et al., 1997). Also, the relative contribution of each coactivator may depend on cell or tissue type and/or coactivator levels in these cells. RAC3/ACTR/AIB-1 is expressed at high levels in placenta, heart, and HeLa cells relative to TIF2 and SRC-1; thus it may serve a more prominent role in nuclear receptor function in these cells (Li and Chen, 1998). In addition, AIB-1 was cloned as a gene that is amplified in ER-positive BT-474, MCF-7, and ZR75 breast cancer cell lines (Anzick et al., 1997). AIB-1 mRNA and protein levels are expectedly higher in these cells as well. SRC-1 and TIF2/GRIP1 are expressed at relatively low levels in these cell lines, suggesting that AIB-1 is specifically involved in the pathogenesis of these tumors. Furthermore, the viability of an SRC-1 knockout mouse may, in part, be due to the observed compensatory overexpression of GRIP1/TIF2 in certain tissues (Xu et al., 1998). RAC3/pCIP levels are unchanged in these tissues compared to the wild-type mouse, again supporting a different functional role for this coactivator versus SRC-1 and TIF2/GRIP1. Finally, a recent study demonstrates that SRC-1 does not colocalize with ER α in rat mammary epithelial cells, but rather is expressed in a distinct subset of cells, suggesting that TIF2/GRIP1 or RAC3/ACTR/AIB-1 may be more important for ER α function in these cells (Shim et al., 1999).

7. Other nuclear receptor coactivators

In addition to the SRC family of coactivators discussed above, many other cofactors have been identified which stimulate the activity of nuclear receptors. For the sake of brevity, we will focus on those associated with SRC coactivators.

7.1. SRA

Recently, in a search for nuclear receptor cofactors, a novel steroid receptor RNA activator (SRA) was isolated in a yeast-two-hybrid screen using the AF-1 domain of the PR as the bait (Lanz et al., 1999). This coactivator is selective for the N-terminal AF-1 activation domain of nuclear receptors and can reverse estrogen-induced squelching of PR-driven gene expression. Interestingly, SRA is also selective for steroid receptors versus RXR heterodimers, for it enhances only PR, GR, AR, and ER activities while having no effect on the activities of the TR β , RAR γ , RXR γ , or PPAR γ . However, maybe the most surprising characteristic of SRA is that it apparently functions as an RNA transcript, which is evident from several observations. First, efforts to generate SRA-encoded protein in vitro or in vivo were not successful. Second, several mutant constructs of SRA that disrupt translational start sites or open reading frames were still able to potentiate PR activity. Third, SRA retained coactivation activity in the absence of protein synthesis via cyclohexamide treatment. Finally, SRA transcripts were identified as components of an SRC-1 complex in vivo via whole-cell fractionation followed by gel filtration chromatography. SRA, detected by RT-PCR, specifically copurified with SRC-1 in the same fractions. SRA mRNA was also efficiently coimmunoprecipitated with SRC-1 antibodies, further supporting the existence of a complex containing SRA and SRC-1. This study suggests that SRA is a novel RNA coactivator that forms a complex with SRC-1 in vivo and selectively enhances the activity of steroid hormone receptors via the AF-1 domain.

7.2. CARM1

A novel enzymatic activity was attributed to nuclear receptor coactivators with the cloning of CARM1 (coactivator-associated arginine methyltransferase 1) via a yeast-two-hybrid screen using the C-terminal amino acids 1121–1462 of GRIP1 (Chen et al., 1999a). This region represents a second, CBP-independent activation domain of GRIP1 (Ma et al., 1999). CARM1 showed extensive homology to the PRMT (protein methyltransferase) family of arginine-specific methyltransferases and interacted with all three members of the SRC coactivator family in vitro. Furthermore, CARM1 contained potent histone methyltransferase activity in vitro, with a preference for histone H3. In vivo, CARM1 enhanced transcription by a Gal4-DBD fusion of GRIP1 1121–1462 and further stimulated GRIP1 coactivation of AR, TR, and ER activities. This coactivation function was dependent upon three amino acids located in the region critical to methyltransferase activity, suggesting that this enzymatic activity is required for CARM1's ability to enhance receptor function. In the

absence of GRIP1, CARM1 had no effect on receptor function, thus SRC coactivators are likely required in order to recruit CARM1 to the receptor complex. Overall, the cloning of CARM1 contributes to the mechanism by which SRC coactivators activate transcription through multiple domains. One activation domain may be required in order to recruit CBP/p300 and histone acetylation activity, while the second activation domain recruits CARM1 and histone methylation activity. These multiple enzymatic functions may be promoter specific or cooperate to remodel chromatin and facilitate transcriptional activation.

7.3. PGC-1

The cloning and characterization of PGC-1 (PPAR gamma coactivator-1) was a critical finding to the field of nuclear receptor coactivators, for it linked coactivator function to the regulation of a specific physiological process, namely adaptive thermogenesis. PGC-1 was isolated in a yeast-two-hybrid screen using PPAR γ 183–505 as the bait and was demonstrated to interact with several members of the nuclear receptor superfamily (Puigserver et al., 1998). It also possesses potent coactivation function for PPAR γ and TR activities at the UCP-1 (uncoupling protein-1) promoter, inducing the expression of this mitochondrial protein involved in heat generation in brown fat cells. Consistently, PGC-1 is also upregulated in muscle and brown fat cells upon exposure to cold temperatures. Further studies have demonstrated that PGC-1 enhances mitochondrial biogenesis and oxygen consumption in muscle cells via the induction of UCP-2 and the regulation of NRFs (nuclear respiratory factors), which are transcription factors that regulate genes involved in mitochondrial DNA replication and transcription (Wu et al., 1999). Finally, a very recent study reports the functional association between PGC-1 and SRC-1 (Puigserver et al., 1999). SRC-1, as well as CBP/p300, interacts with PGC-1 in vitro and in vivo and enhances transcriptional activation by a Gal4DBD fusion of PGC-1 in transient transfection assays. These interactions are mediated by SRC-1 782–1139 and p300 1805–2441. Intriguingly, expression of PPAR γ or NRF-1 also enhanced Gal-PGC-1 activity, while cotransfection of PPAR γ increased the interaction between PGC-1 and SRC-1 or CBP/p300 in vitro and in vivo. These data support a model of PGC-1 activation in which the interaction with a transcription factor such as PPAR γ stimulates PGC-1 activity by inducing SRC-1 recruitment (Puigserver et al., 1999). This recruitment likely is the result of a conformational change in PGC-1 that occurs upon binding to the transcription factor. This study also suggests the SRC coactivator function may be essential to adaptive thermogenesis, for it may be required for transcriptional activation by PGC-1.

8. DRIP/TRAP coactivator complexes

Much effort has been made recently to isolate and purify an entire complex of polypeptides that functions to coactivate nuclear receptor function. To this end, several groups have identified virtually identical complexes that appear distinct from the SRC coactivator complex. Using the VDR-LBD as an affinity matrix, a complex was purified from Namalwa cell extracts termed DRIP (VDR-interacting proteins) that specifically interacts with ligand-bound VDR-LBD (Rachez et al., 1998). The same complex was also purified using affinity chromatography from HeLa cells that constitutively express Flag-tagged TR and named TRAP (TR-associated proteins) (Fondell et al., 1996). Subsequently, this complex was identified as ARC (activator-recruited cofactor) (Naar et al., 1999) and SMCC (Srb/Mediator coactivator complex) (Gu et al., 1999). The DRIP/TRAP complex lacks CBP/p300 or SRC proteins (Rachez et al., 1999) and is recruited to the receptor AF-2 domain by the DRIP205/TRAP220 subunit via a single LXXLL motif (Rachez et al., 1999; Yuan et al., 1998). Unlike SRC coactivators, the DRIPs/TRAPs have been demonstrated to be required

for transcriptional activation by nuclear receptors in cell-free *in vitro* transcription assays (Rachez et al., 1998; Fondell et al., 1996). The DRIPs also enhance VDR activity on chromatin-organized templates despite a lack of HAT activity, suggesting a potential unidentified chromatin remodeling function (Rachez et al., 1999). Furthermore, it is evident that this complex plays a more global role in transcriptional activation rather than being specific to nuclear receptors, for ARC was identified as a coactivator for VP16 and p65 (Naar et al., 1999), while SMCC enhances p53 activity (Gu et al., 1999). What is not clear is the mechanism by which the DRIP/TRAP and SRC complexes both contribute to overall nuclear receptor function. One possibility involves a two-step model in which the SRC complex is first recruited to the nuclear receptor to open up the chromatin network via histone acetylation (Freedman, 1999). This would allow access for the large DRIP/TRAP complex, which would subsequently remodel chromatin, facilitating the organization of the pre-initiation complex or binding of other transcription factors. However, it is also possible that the DRIPs/TRAPs may target RNA polymerase to the target gene promoter, for several subunits are homolo-

Model of SRC coactivator function

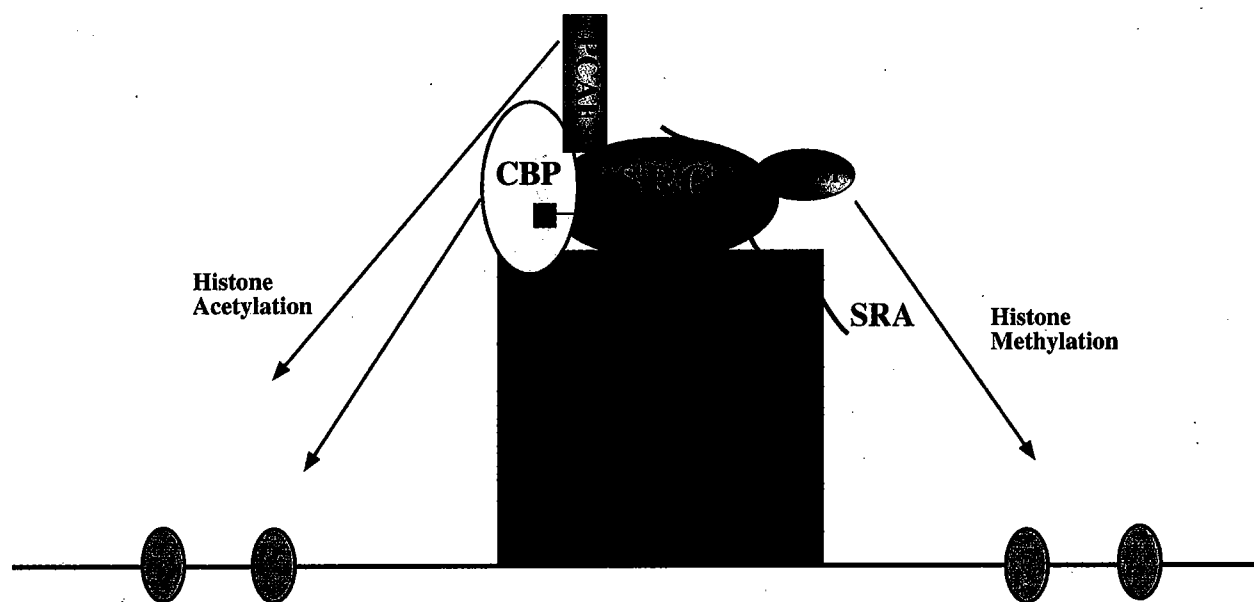


Fig. 2. Model of SRC coactivator function. The nuclear receptor is able to recruit an SRC coactivator upon binding hormone, which subsequently results in the recruitment of additional coactivators to the complex. SRC is able to interact with the receptor and CBP/p300 via LXXLL nuclear receptor (NR) boxes. The histone acetylation and methylation activities of various constituents of the coactivator complex facilitate the relaxation of the chromatin architecture at the target gene promoter, thereby enhancing transcriptional activation. It should be noted that this is only a general model of the coactivator complex. It is likely that additional cofactors are involved and that different receptors may recruit different components of the complex, thus achieving a level of specificity among receptors and coactivators. NR=nuclear receptor, SRC=steroid receptor coactivator, SRA=steroid receptor RNA activator, CARM=coactivator associated arginine methyltransferase, CBP=CREB-binding protein, PCAF=p300/CBP-associated factor.

gous to proteins found in Mediator, a transcriptional regulatory complex that associates with RNA pol II (Kim et al., 1994). In support of this, RNA pol II can be isolated with the SMCC complex at low ionic strength (Gu et al., 1999). One also cannot rule out the possibility that SRC and DRIP/TRAP functions are not integrated at all, but rather have cell-type, promoter, or transcription factor specificity. Specificity may also be the result of the alteration of one or more of the subunits of the complex, depending on the target gene. Overall, it is clear that the DRIP/TRAP complex is likely involved in the regulation of a broad range of signaling pathways, but whose biological role is unknown.

9. Model of SRC function

In order to integrate the wealth of data collected on the mechanism of action of SRC coactivators, we propose the following model of SRC function in the regulation of nuclear receptor activity (Fig. 2). Hormone binding triggers nuclear translocation of Type I steroid receptors and the release of the corepressor complex from Type II non-steroid receptors and subsequent recruitment of an SRC coactivator to the target gene promoter. SRC is able to interact with the AF-2 domain of each monomer of the dimer via multiple, α -helical NR boxes located in the receptor-interacting domain. SRC is likely complexed with the RNA coactivator SRA, which enhances AF-1 activity. After initial SRC docking to the receptor, it is able to recruit additional coactivators to the complex. These include CBP/p300, which uses the NR boxes of the SRC transcriptional activation domain for interaction with coactivator, and the CBP/p300-associated factor P/CAF. Additional, direct interactions between CBP/p300 and nuclear receptors and between P/CAF and SRC have also been reported, which may enhance complex formation. Furthermore, SRC is also able to recruit CARM1 to the target gene via a different domain than that required for CBP/p300 binding. Once this complex is assembled, the histone acetylase activities of CBP/p300, P/CAF, and possibly SRC itself, together with the histone methyltransferase activity of CARM1, serve to remodel the chromatin architecture, thus facilitating the access of additional transcription factors, coactivators such as the DRIP/TRAP complex, and/or the basal transcription machinery to the target gene promoter to activate transcription. Of course, caveats to this model likely exist. For example, the coactivator complex may be comprised of different components depending on the specific nuclear receptor, cell type, or target gene. Different coactivator complex components may create a level of specificity among different receptors that answers the questions surrounding the potential redundancy among the members of the SRC family. Also, with the

intense focus on hormone action and plethora of receptor cofactors being cloned in recent years, it is likely that additional members of the coactivator complex have yet to be identified. In addition, non-histone substrates for the enzymatically active cofactors may be involved, for as described above, CBP/p300 can acetylate non-histone proteins such as ACTR, p53, and TFIIE/TFIIF. Finally, it is possible that the receptor is able to recruit single, pre-formed coactivator complex to the target gene upon hormone binding. However, though the precise details of transcriptional activation by nuclear receptors are still not clear, it is evident that the SRC family of coactivators is critical to receptor function and will continue to warrant investigation into its role in intracellular signaling pathways.

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Sequestration and Inhibition of Daxx-Mediated Transcriptional Repression by PML

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PML fuses with retinoic acid receptor α (RAR α) in the t(15;17) translocation that causes acute promyelocytic leukemia (APL). In addition to localizing diffusely throughout the nucleoplasm, PML mainly resides in discrete nuclear structures known as PML oncogenic domains (PODs), which are disrupted in APL and spinocellular ataxia cells. We isolated the Fas-binding protein Daxx as a PML-interacting protein in a yeast two-hybrid screen. Biochemical and immunofluorescence analyses reveal that Daxx is a nuclear protein that interacts and colocalizes with PML in the PODs. Reporter gene assay shows that Daxx drastically represses basal transcription, likely by recruiting histone deacetylases. PML, but not its oncogenic fusion PML-RAR α , inhibits the repressor function of Daxx. In addition, SUMO-1 modification of PML is required for sequestration of Daxx to the PODs and for efficient inhibition of Daxx-mediated transcriptional repression. Consistently, Daxx is found at condensed chromatin in cells that lack PML. These data suggest that Daxx is a novel nuclear protein bearing transcriptional repressor activity that may be regulated by interaction with PML.

Acute promyelocytic leukemia (APL) arises as a result of chromosomal translocation involving the retinoic acid (RA) receptor α (RAR α) gene on chromosome 17 fused with either the promyelocytic leukemia gene (PML) on chromosome 15, the promyelocytic leukemia zinc finger gene (PLZF) on chromosome 11, the nucleophosmin/B23 (NPM) gene on chromosome 5, or the nuclear mitotic apparatus gene (NuMA) on chromosome 11 (30, 39). The t(15;17) translocation between PML and RAR α accounts for nearly all APL cases. This translocation creates an oncogenic fusion protein, PML-RAR α , which contains both the DNA-binding domain (DBD) and ligand-binding domains of RAR α and the N terminus of PML. Transgenic mice that overexpress PML-RAR α or PLZF-RAR α developed an APL-like phenotype (9, 21, 26), suggesting that these fusion proteins are directly involved in APL pathogenesis. Recent studies have focused on analyzing the functional properties of PML-RAR α and PLZF-RAR α (20, 22, 25, 40) in order to understand the molecular basis of leukemogenesis. Both fusion proteins form homodimers that bind to RA response elements and interact with the nuclear receptor corepressors SMRT (silencing mediator for retinoid and thyroid hormone action) and N-CoR (nuclear receptor corepressor), which in turn recruit a histone deacetylase complex (1, 27, 40, 46). Pharmacological concentrations of all-*trans*-RA (atRA) induce dissociation of the corepressors from PML-RAR α , but not PLZF-RAR α , due to the presence of an additional, RA-insensitive corepressor-interacting surface on PLZF. This differential degree of dissociation of corepressors induced by atRA correlates with the ability of histone deacetylase inhibitors and atRA to induce terminal differentiation of these two subtypes of APL cells. These findings indicate that abnormalities in transcriptional repression by the oncogenic fusion proteins may be involved in leukemogenesis.

PML belongs to a family of proteins characterized by the presence of a RING finger domain (8). RING finger proteins are implicated in transcriptional regulation, and some members of the RING family are associated directly with chromatin (53). Ablation and overexpression experiments suggest an important role of PML in the regulation of cell growth, hematopoietic cell differentiation, tumorigenesis, apoptosis, and RA signaling (44, 63). In normal cells, PML is concentrated within 10 to 20 nuclear structures known as nuclear domains 10 (ND10), Krüppel bodies, nuclear bodies, or PML-oncogenic domains (PODs) (2, 17, 33, 59, 65). The POD structure is disrupted in the t(15;17) translocated APL cells (17, 33, 65), presumably through interaction of wild-type PML with PML-RAR α . Interestingly, the POD structure reorganizes upon treatment with atRA or arsenic trioxide (As₂O₃), a process that correlates with differentiation of APL cells, indicating that the POD structure might affect promyelocyte differentiation.

In addition to PML, the POD contains several other proteins, including the 100-kDa nuclear protein antigen (Sp100) (2), the small ubiquitin-related modifier (SUMO-1 [41], also known as PML-interacting clone 1 [PIC1] [7], ubiquitin-like 1 [UBL1] [57], or sentrin [48]), and the 140-kDa protein (Sp140) (6). Sp100 is a nuclear antigen recognized by autoantibodies from patients with primary biliary cirrhosis (62). Expression of both PML and Sp100 are upregulated by interferon (23). SUMO-1 was recently identified as a ubiquitin-like protein that forms covalent conjugates with PML and Sp100 (7, 58). In addition, the CREB-binding protein (CBP) and the retinoblastoma tumor suppressor (pRB) have been found in the PODs (35, 61). Also, the PODs are targets of several viral proteins, which alter POD structure (11, 14, 18). Although there is evidence for POD's role in transcriptional activation (15, 35), DNA replication (19), apoptosis (51, 64), and viral infection (14, 42), the precise function of PODs in these processes remains unclear.

We have sought to understand the function of PODs through identification of PML-interacting proteins that also localize in the PODs. By using the yeast two-hybrid system, we identified SUMO-1 and the Fas-binding protein Daxx (68)

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(J. D. Chen and R. M. Evans, unpublished data). Daxx has been shown to promote Fas-mediated apoptosis through activation of the Jun NH₂-terminal kinase (JNK) and JNK kinase ASK1 (apoptosis signal-regulating kinase 1) (12). Recent data suggest that Daxx is not sufficient for Fas-mediated apoptosis, since a Fas mutant that selectively binds to Daxx but not the Fas-adaptor death domain-containing protein (FADD/MORT1) failed to induce apoptosis (13). Other evidence suggests that Daxx may interact with the centromeric protein-c (CENP-C) and may bind to a steroidogenic factor 1 (SF-1)-like DNA element (32, 50). Therefore, the exact mechanism by which Daxx regulates Fas-mediated apoptosis may involve nuclear processes.

In the present study, we have characterized both biochemical and functional interactions between Daxx and PML. Daxx resides primarily in the cell nucleus, where it forms a complex with PML. Confocal immunofluorescence data demonstrate that Daxx colocalizes with PML in the PODs, and such colocalization persists in NB4 APL cells (36) before and after treatment with atRA and As₂O₃. Daxx possesses strong transcriptional repressor activity and appears to interact directly with histone deacetylases. Intriguingly, overexpression of PML inhibits Daxx-mediated transcriptional repression and, in cells that lack PML, Daxx is preferentially associated with condensed chromatin. Our data reveal a new role for Daxx in transcriptional repression and suggest a novel function of PML and the POD structure in the suppression of transcriptional repression.

MATERIALS AND METHODS

Yeast two-hybrid system. The screening of PML-interacting proteins was conducted by the yeast two-hybrid system by using the Y190 strain as previously described (16). The Gal4 DBD (amino acids 1 to 147) fusion of full-length PML (29) was constructed in the yeast vector pAS1 (16). The resulting Gal4 DBD-PML fusion protein was used as bait to screen a Gal4 activation domain (AD)-fused human B-lymphocyte cDNA library in the pACT expression vector (16). About 10⁶ yeast transformants were screened on selection plates containing 50 mM 3-aminotriazole (Sigma). For ligand treatment, the culture was incubated in the presence of ligand or solvent (control) for 24 h before measuring the β -galactosidase (β -Gal) activity.

Biochemical cell fractionation. HeLa cells (2×10^6) were harvested into 500 μ l of CLB buffer (10 mM HEPES, 10 mM NaCl, 1 mM KH₂PO₄, 5 mM NaHCO₃, 1 mM CaCl₂, 0.5 mM MgCl₂)-5 mM EDTA-1 mM phenylmethylsulfonylfluoride-proteinase inhibitors. Cells were allowed to swell for 5 min on ice, Dounce homogenized 35 times, and centrifuged at 7,500 rpm for 5 min to pellet nuclei and debris. The supernatant (cytosol plus plasma membrane) was then spun at 25,000 rpm for 30 min to pellet the membrane. The nucleus-debris pellet was resuspended in 1 ml of TSE buffer (10 mM Tris, pH 7.5; 300 mM sucrose; 1 mM EDTA) and Dounce homogenized 30 times, followed by centrifugation at 5,000 rpm for 5 min. The pellet was resuspended and washed twice to obtain the final nucleus pellet. Equal amounts of protein in each fraction were analyzed Western blotting.

Western blotting. Western blotting was conducted by using the enhanced chemiluminescence reagents according to the manufacturers' recommendation (Amersham). The affinity purified anti-Daxx polyclonal antibodies were raised against glutathione *S*-transferase (GST)-Daxx (amino acids 556 to 740) fusion protein and subsequently purified with the GST-Daxx protein column as described earlier (24). Anti-Gal4-DBD antibody was purchased from Santa Cruz, and anti-HDAC1 antibody was from Upstate Biotechnology.

Co-IP. Coimmunoprecipitation (Co-IP) was conducted according to a standard procedure by using the protein A-agarose beads (Santa Cruz) (24). Nuclear extracts were prepared as described earlier (3). HeLa and NB4 cells were lysed in cell lysis buffer (0.4 M NaCl, 0.2 mM EGTA, 10% glycerol, 1% NP-40), and cell extracts were precleared by incubating them with protein-A agarose beads for 1 h at room temperature. The affinity-purified IP antibodies were conjugated with protein A-agarose beads in cell lysis buffer for 2 h at room temperature. The antibody-protein A-agarose was collected by brief centrifugation and incubated with cell extracts (100 μ g) overnight at 4°C. The precipitates were collected by centrifugation and washed five times with excess phosphate-buffered saline containing 0.1% NP-40. The final precipitate was dissolved in sodium dodecyl sulfate (A) (SDS) sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting.

Immunofluorescence and confocal microscopy. Cells were grown on cover glasses (VWR Scientific), fixed in a methanol-acetic acid (1:1) mixture on dry ice

for 2 min and processed for immunofluorescence staining as described elsewhere (17). For NB4 cells, the cover glasses were coated with poly-L-lysine before seeding the cells. After immunostaining, cell nuclei were stained with DAPI (4',6-diamidino-2-phenylindole dihydrochloride hydrate) (Sigma). Confocal microscopy was conducted with a Leica TCS SP spectral laser scanning confocal microscope. Channel cross-talk was avoided by reducing the intensity of the excitation laser beam in the absence of the other excitation laser. Standard epifluorescence microscopy was performed on an Olympus IX-70 microscope equipped with a back-illuminated cool charge-coupled device (CCD) camera (Princeton Instruments), and the image was processed by using the MetaMorph software (Universal Imaging Corp.).

Transient-transfection assay. Transient transfection was conducted using a standard calcium phosphate precipitate method as described earlier (3). Cultured cells were maintained in Dulbecco modified Eagle medium or RPMI medium (for NB4 cells) supplemented with 10% fetal bovine serum (Gibco). Twelve hours prior to transfection, 2×10^4 cells were plated in each well of 12-well plates. Transfected cells were refed with fresh media and harvested 36 to 48 h after transfection. Transfected cells in each well were lysed and processed for luciferase and β -Gal assay as described elsewhere (38). The luciferase activity was determined with an MLX plate luminometer (Dynex) and normalized with the cotransfected β -Gal.

Far-Western blot. GST fusion proteins were expressed in DH5 α cells and purified by standard glutathione agarose beads according to manufacturer's recommendation (Pharmacia). The purified proteins were separated by SDS-PAGE and electroblotted onto a nitrocellulose filter in transfer buffer (25 mM Tris-HCl, pH 8.3; 192 mM glycine; 0.01% SDS). Proteins were denatured with 6 M guanidine hydrochloride (GnHCl) and renatured by stepwise dilution of GnHCl. Filters were blocked and hybridized overnight with ³⁵S-labeled protein as described elsewhere (38). The membrane was then washed three times with hybridization buffer, and the bound probe was detected by autoradiography.

GST pull-down assay. The GST pull-down assay was conducted according to a protocol as described earlier (24). Briefly, 5 μ g of glutathione agarose-protein beads was incubated with 5 μ l of in vitro-translated ³⁵S-labeled protein with moderate shaking at 4°C overnight in binding buffer (20 mM HEPES, pH 7.7; 75 mM KCl; 0.1 mM EDTA; 2.5 mM MgCl₂; 0.05% NP-40; 1 mM dithiothreitol; 1 mg of bovine serum albumin per ml). The bound protein was washed three times with the binding buffer, and the beads were collected by centrifugation. The bound protein was eluted in SDS sample buffer and analyzed by SDS-PAGE and autoradiography.

Site-directed mutagenesis. Site-directed mutagenesis was conducted by using the Quick-Change site-directed mutagenesis kit according to manufacturer's instruction (Stratagene). A mammalian hemagglutinin (HA)-PML vector was used as a template, and mutagenesis was conducted in three rounds consecutively on the same template. The mutated construct was confirmed by DNA sequencing by using dideoxynucleotide chain-termination reactions and Sequenase (U.S. Biochemicals).

RESULTS

Identification of Daxx as a PML-interacting protein. In the yeast two-hybrid screen, we identified a PML-interacting clone that encodes the C-terminal 184 amino acids of Daxx (32, 50). Yeast two-hybrid assay shows that this Daxx clone interacts with Gal4 DBD fusions of both PML and PML-RAR α but not SP100 (Fig. 1A), suggesting that Daxx may be a PML-interacting protein. Since atRA binds to PML-RAR α in a way similar to that of wild-type RAR α (4), we determined the effect of atRA on interaction between Daxx and PML-RAR α (Fig. 1B). atRA inhibits the two-hybrid interaction between Daxx and PML-RAR α efficiently and in a dose-dependent manner. The inhibition of binding is slightly more sensitive with the long form of PML-RAR α than with the short form, a finding consistent with the higher affinity of the long form of PML-RAR α for atRA (4). This atRA-dependent inhibition of binding is specific, for atRA has no effect on the interaction between PML and Daxx while it enhances the interaction between PML-RAR α and the coactivator RAC3 (38). Also, the thyroid hormone triiodothyronine that does not bind PML-RAR α also has no effect on the interaction between Daxx and PML-RAR α . These data suggest that Daxx is a PML-interacting protein that may also associate with the oncoprotein PML-RAR α in the absence of atRA.

Daxx forms a complex with PML in vivo. In addition to being diffusely distributed in the cytoplasm, PML is mainly a nuclear protein, while Fas is a transmembrane cell surface

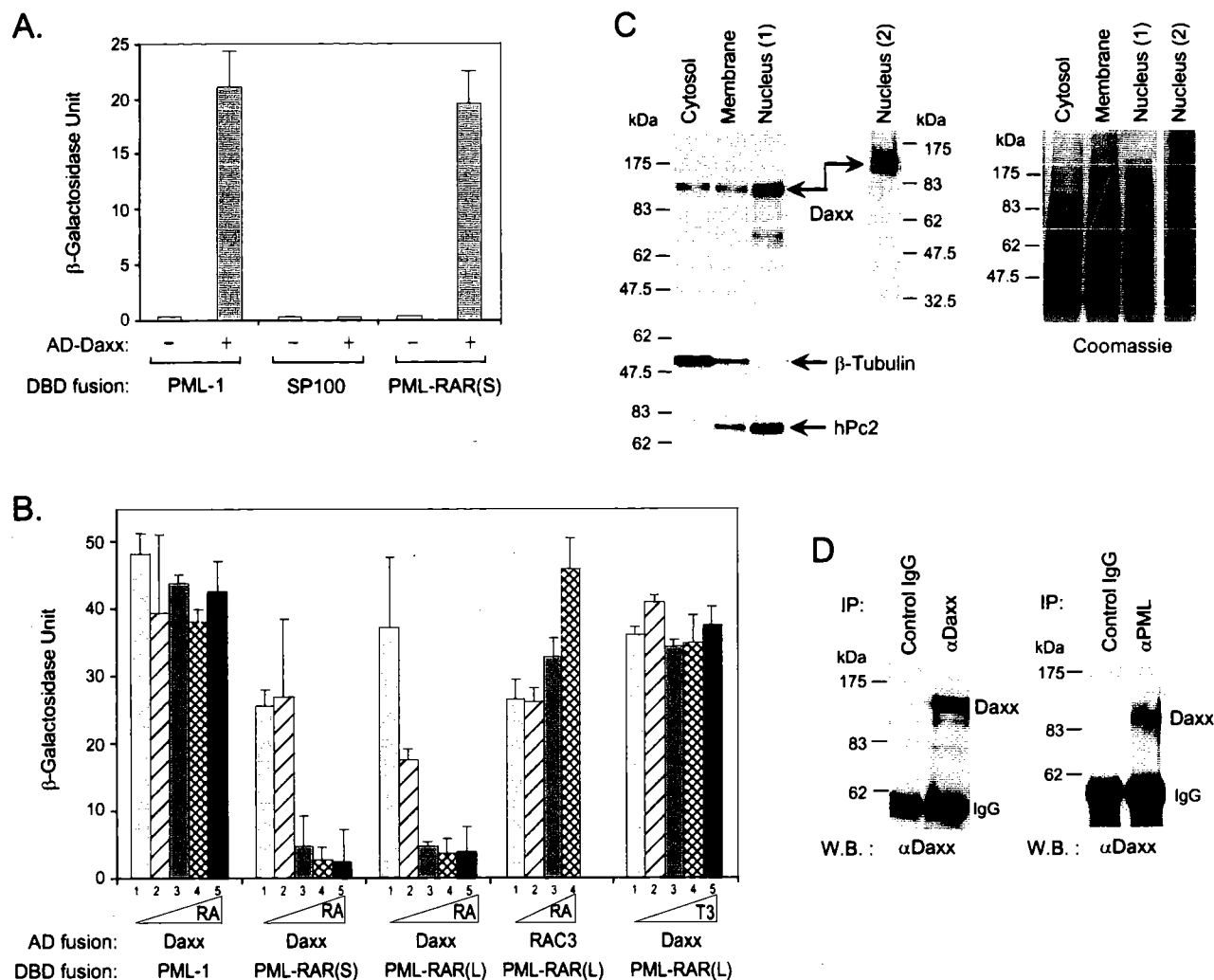


FIG. 1. Interaction between Daxx and PML in vivo. (A) Interaction of Daxx with PML in yeast two-hybrid system. The average β -Gal activities of three transformants expressing the indicated combinations of Gal4 AD and DBD fusion proteins were determined as described in Materials and Methods. The AD-Daxx fusion protein contains amino acids 556 to 740 of human Daxx. The DBD fusion proteins contain full-length PML-1, SP100, and PML-RAR α short form, respectively. The minus sign indicates empty vector alone. (B) atRA disrupts the interaction between Daxx and PML-RAR α . The effect of atRA on Daxx-PML-RAR interaction was determined after a 24-h incubation of the culture in the presence of the indicated concentrations of respective ligands. Columns: 1, solvent only; 2, 1 nM; 3, 10 nM; 4, 100 nM; and 5, 1,000 nM. T3, 3,3',5-triiodo-L-thyronine. (C) Subcellular fractionation of Daxx. HeLa cells were fractionated into cytosolic, membrane, and nuclear fractions, and an equal amount of protein was analyzed by Western blotting for Daxx (left panel). The distribution of the cytoplasmic protein β -tubulin and the nuclear protein hPc2 in each fraction was also determined by immunoblotting to validate the fractionation. Two independent preparations of HeLa nuclear extracts are shown. The right panel is a Coomassie blue-stained gel that shows the relative amount of proteins in each fraction used in the Western blot. (D) Co-IP of Daxx with PML. NB4-cell extracts were immunoprecipitated with affinity-purified anti-Daxx and anti-PML antibodies, and the presence of Daxx in the immunoprecipitates was determined by immunoblotting with anti-Daxx antibodies. The antibodies used for the IP and the Western blot (W.B.) are indicated.

receptor. Since Daxx interacts with both PML and Fas, it is important to determine whether Daxx is a nuclear or cytoplasmic protein. We analyzed the subcellular distribution of Daxx by using biochemical fractionation followed by Daxx immunoblotting. In this assay, Daxx cofractionates primarily with nuclear fraction, with a minority also present in the cytosolic and membrane fractions (Fig. 1C). Control antibodies against the cytoplasmic protein β -tubulin and the nuclear protein polycomb hPc2 (55) show no cross-contamination between the cytoplasmic and nuclear fractions. All of these proteins were detected in the membrane fraction, presumably because this fraction also contains insoluble organelles involved in protein synthesis and transportation. These results demonstrate that Daxx resides mainly in the cell nucleus, suggesting that Daxx may interact with PML in the nucleus.

To confirm that the interaction between Daxx and PML also occurs in mammalian cells, we performed Co-IP assays from HeLa and NB4 cell extracts (Fig. 1D). Both anti-Daxx and anti-PML antibodies, but not preimmune serum, efficiently coimmunoprecipitate endogenous Daxx. These data suggest that Daxx may form a stable complex with PML in vivo. In the immunoprecipitates of Daxx and PML antibodies, we also detected weak signals of the 90-kDa PML and two SUMO-1-conjugated forms of PML (data not shown), confirming the presence of PML in the IP. We also attempted to demonstrate an interaction between Daxx and PML in vitro in GST pull-down and far-Western assays, but all experiments failed to show a convincing interaction. We reasoned that this might be due to the fact that PML is extensively modified by SUMO-1 in

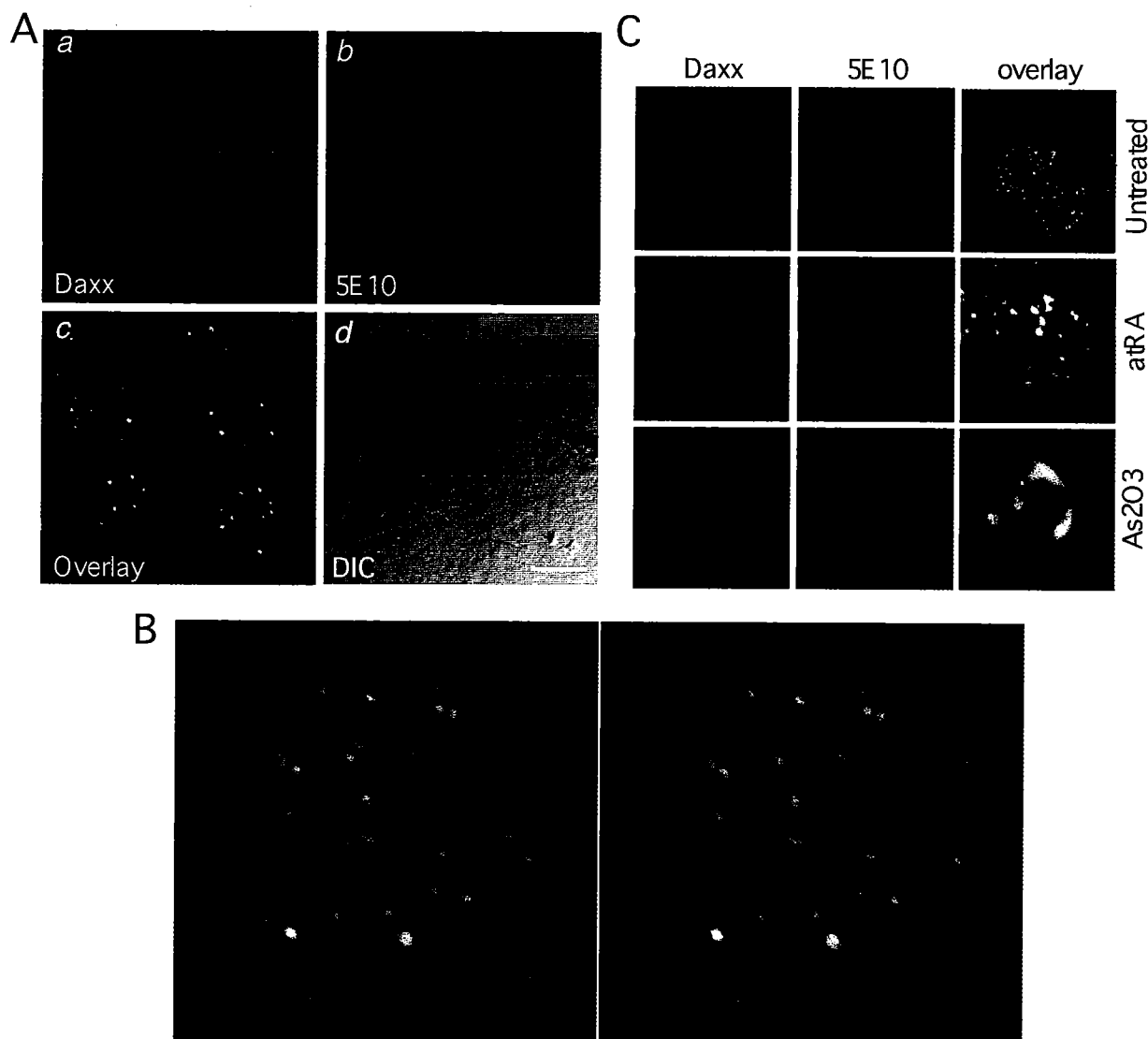


FIG. 2. Daxx colocalizes with PML at the PODs. (A) Confocal immunofluorescence analysis of endogenous Daxx and PML. HEP2 cells were fixed and immunostained with affinity-purified rabbit anti-Daxx polyclonal antibodies and mouse anti-PML 5E10 monoclonal antibodies as described in Materials and Methods. The sample was analyzed by use of a confocal microscope. Panels a and b show the signals of Daxx (green) and PML (red) on a single confocal section. Panel c shows colocalization (yellow signals) of Daxx and PML in the merged image. Panel d is a differential interference contrast image showing the surfaces of the cells and nuclei. Bar, 10 μ m. (B) Three-dimensional presentation of the colocalization between Daxx and PML. Total of 32 consecutive z-sections at increments of 0.08 μ m were reconstructed into a three-dimensional image by using the Leica confocal software. The right and left projected images were rotated 4.5° at opposite directions along the x (horizontal) axis. Yellow represents the colocalization between Daxx (green) and PML (red). (C) Colocalization of Daxx and PML in APL cells. NB4 cells were plated on cover glasses coated with poly-L-lysine. The control (untreated), atRA-treated (1 μ M for 72 h), and As₂O₃-treated (1 μ M for 72 h) cells were fixed and immunostained with anti-Daxx polyclonal and anti-PML monoclonal antibodies. Colocalization of Daxx and PML was revealed by confocal laser microscopy (except for the untreated cells).

vivo (44, 45, 58) or that an additional factor may bridge the interaction between PML and Daxx.

Daxx colocalizes with PML in the PODs. We then wished to determine if Daxx colocalizes with PML in the PODs in order to provide further evidence for a physiological interaction between Daxx and PML. Confocal immunofluorescence microscopy using affinity-purified anti-Daxx antibodies reveals discrete nuclear structures in interphase HEP2 cells, in addition to an evenly distributed nucleoplasmic staining (Fig. 2Aa). Double immunostaining, together with use of anti-PML antibodies, demonstrates that the Daxx foci colocalize perfectly with the PODs in cell nuclei (Fig. 2Aa to d). Such colocalization occurs in many different cell types, including HeLa, HEK293, and A549 cells and normal human fibroblasts, sug-

gesting that colocalization between Daxx and PML may be a common phenomenon in different cell types. The colocalization has been confirmed by using antibodies against different POD antigens, including SP100 and SUMO-1, as well as under conditions that modify the POD structure, such as with interferon, As₂O₃ treatments, and viral infections (unpublished data). A three-dimensional topographic analysis of the colocalization between Daxx and PML demonstrates an extensive colocalization between Daxx and PML in the PODs (Fig. 2B).

Colocalization of Daxx and PML in NB4 APL cells. We next analyzed the distribution of Daxx in the NB4 APL cells (Fig. 2C), in which the PODs are disrupted into "microparticulate" structures. Similar to PML, Daxx is also disrupted in the NB4 cells, in which it remains colocalized with PML. The presence

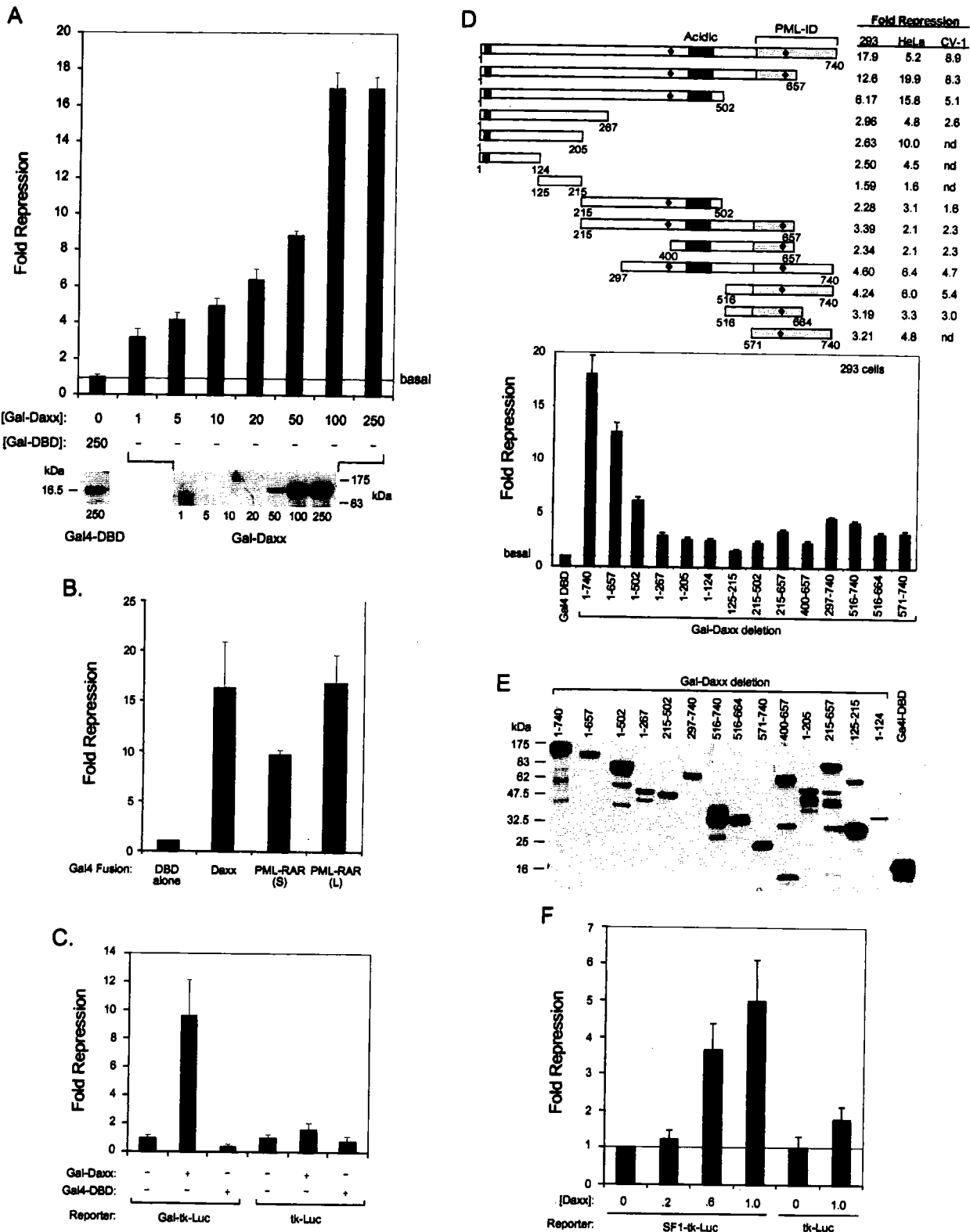


FIG. 3. Modulation of promoter activity by Daxx. (A) Transcriptional repression by Gal-Daxx. Recruitment of Daxx to a promoter via Gal4-DBD results in inhibition of basal transcription in a dose-dependent manner. Transient transfection was conducted in HEK293 cells with increasing concentrations (nanograms) of Gal-Daxx as indicated. The relative fold repression of the basal promoter activity in the presence of Gal-Daxx was compared to that of Gal4-DBD alone. The bottom panels show immunoblots with anti-Gal4-DBD antibodies of the transfected fusion protein at indicated concentrations of expression vector. (B) Daxx represses basal transcription as strong as PML-RAR α . HEK293 cells were transfected with equal amounts (250 ng) of each expression vector, and the relative repression was determined as described in Materials and Methods. The results show that Gal-Daxx represses basal transcription as strongly as Gal-PML-RAR α . (C) Requirement of binding sites for transcriptional repression by Gal-Daxx. HEK293 cells were transfected with 250 ng of Gal-Daxx or Gal4-DBD alone, and the effects on the promoter activities of Gal-tk-luciferase (luc) and tk-luc reporters were determined. The Gal-tk-luc reporter contains four copies of Gal4-binding sites in front of the minimal tk promoter,

of PML-RAR α fusion protein in the microparticulate structures (17) supports the observed interaction between Daxx and PML-RAR α (Fig. 1). Upon atRA treatment, PML-RAR α is degraded in NB4 cells (47), and these microparticulate structures reorganize into normal size of the PODs (17, 65), where Daxx and PML remain colocalized. The colocalization between Daxx and PML is more evident in NB4 cells treated with As₂O₃, in which larger and fewer PODs are observed. These results suggest that Daxx and PML colocalize in APL NB4 cells, and such colocalization persists after reorganization of the PODs induced by atRA or As₂O₃.

Daxx represses basal transcription. Several POD-associated proteins, including PML, are implicated in transcriptional regulation (for reviews see references 34 and 39). Since Daxx interacts with PML and localizes at the PODs, we decided to test whether Daxx might regulate transcription. Transfection of the Gal4-DBD full-length Daxx fusion protein (Gal-Daxx) in HEK293 cells strongly inhibits basal transcription of the Gal4-tk-luciferase reporter in a dose-dependent manner (Fig. 3A, top). Western blotting using anti-Gal4 DBD antibodies confirms increased expression of Gal-Daxx in transfected cells in the presence of higher concentrations of DNA (Fig. 3A, bottom). Comparison of Daxx-mediated transcriptional repression with that of PML-RAR α fusion proteins indicates that Daxx represses as strongly as the PML-RAR α oncoprotein (Fig. 3B). Moreover, repression by Gal-Daxx requires Gal4-binding sites (Fig. 3C) and occurs in multiple cell types (Fig. 3D), demonstrating the specificity of the observed Daxx-mediated transcriptional repression.

We attempted to determine the sequences in Daxx that are responsible for the repression activity by standard deletion analysis (Fig. 3D). Progressive deletion from the C terminus to residue 124 gradually reduces the repression activity of Daxx in a cell-type-dependent manner. Deletions of the N terminus and several other mutants also show a significant decrease in repression. Equal expression of these Gal-Daxx deletion proteins in transfected cells is confirmed by Western blotting using the anti-Gal4 DBD antibodies (Fig. 3E). These data suggest that multiple regions of Daxx may be important for transcriptional repression in a cell-type-dependent manner.

Daxx was previously isolated in a yeast one-hybrid screen using a reporter containing a SF-1-like element (32). We decided to investigate whether Daxx can repress transcription from a promoter containing the SF-1-like element in a transient-transfection assay (Fig. 3F). As expected, overexpression of wild-type Daxx represses basal transcription from the SF1-tk-luciferase reporter that contains four copies of the SF1-like element, while it has little effect on the tk-luciferase reporter lacking the SF-1 sites. These data indicate that Daxx may repress the basal transcription of natural promoters containing SF1-like elements, a result consistent with the strong repressor activity observed with the Gal-Daxx fusion protein.

Daxx interacts with HDACs. Histone deacetylation has been demonstrated to play a central role in transcriptional repression by inducing chromatin assembly and condensation (49, 66). To determine whether histone deacetylation is required

for Daxx-mediated transcriptional repression, we analyzed the interaction between Daxx and the three available human histone deacetylases (HDACs) (67). The three human HDACs are highly conserved in structure and function. All of them repress basal transcription in the Gal4-DBD fusion assay, and all display histone deacetylase activity (67). Far-Western analyses demonstrate interactions between Daxx and all three GST-HDAC fusion proteins, but not GST alone, while PML and SP100 show no interaction with any of these GST-HDACs under the assay conditions (Fig. 4A and B and data not shown). A positive control shows that PML interacts efficiently with GST-PML under identical conditions (Fig. 4C). Furthermore, a Daxx mutant (amino acids 400 to 657) that possesses weak repression activity also does not interact with HDAC1 (Fig. 4C). These data support a role for HDAC interaction in Daxx-mediated transcriptional repression. The interaction between Daxx and HDAC1 is further confirmed in a GST pull-down assay (Fig. 4D), in which GST-HDAC1, but not GST alone, precipitates about 20% of input ³⁵S-labeled Daxx. Moreover, an interaction between Daxx and HDAC1 *in vivo* is also observed by Co-IP of HeLa nuclear extracts (Fig. 4E), in which HDAC1 coimmunoprecipitates with Daxx antibodies but not with the preimmune serum. Together, these experiments provide strong evidence that Daxx and HDACs interact *in vitro* and *in vivo*.

HDAC inhibitor reverses Daxx-mediated repression. The physical interaction observed between Daxx and HDAC suggests that Daxx may recruit a HDAC corepressor complex to repress basal transcription via histone deacetylation and chromatin condensation. To provide more evidence for this possibility, we assayed the effect of a histone deacetylase inhibitor, trichostatin A (TSA), on the repressor activity of Gal-Daxx in a transient-transfection assay (Fig. 4F). As expected, TSA reverses transcriptional repression by Gal-Daxx in a dose-dependent manner, while it has little effect on Gal4-DBD alone under identical conditions. These data indicate that histone deacetylation is involved in transcriptional repression by Daxx.

Inhibition of Daxx-mediated transcriptional repression by PML. Since Daxx was identified as a PML-interacting protein and subsequently demonstrated to possess strong transcriptional repression activity, we decided to investigate the role of PML in the regulation of transcriptional repression by Daxx. To do this, Gal-Daxx was cotransfected with increasing amounts of full-length PML into HEK293 cells and the activity of the luciferase reporter was measured (Fig. 5A). As observed above, Gal-Daxx represses reporter expression strongly when compared to the Gal4-DBD alone (Fig. 5A, compare lanes 1 and 6). Interestingly, coexpression of increasing amounts of PML inhibits this repression in a dose-dependent manner, abolishing nearly all of the repressor function of Gal-Daxx (lanes 2 to 5). This effect is specific to Gal-Daxx, for cotransfection of PML with the Gal4-DBD alone has little effect on reporter activity (lanes 7 and 8). These data suggest that PML may inhibit Daxx-mediated transcriptional repression.

Similar experiments were then performed to determine if PML-RAR α might also regulate the function of Gal-Daxx.

while the tk-luc lacks the binding sites. (D) Mapping of the Daxx sequences required for repression. Schematic presentation of Gal-Daxx deletion mutants and their effects on promoter activity in HEK293, HeLa, and CV-1 cells are summarized. The two acidic regions are indicated by black bars, and the two potential nuclear localization signals are marked with diamonds. The bottom graph shows a column presentation of the repression activity of various Gal-Daxx deletion mutants in HEK293 cells. (E) Expression of Gal-Daxx mutants in transfected cells. The transfected lysates were analyzed by immunoblotting by using mouse anti-Gal4-DBD monoclonal antibodies. The top band in each lane represents the expected molecular weights of the Gal-Daxx mutants, except for Gal-Daxx (125-215), where the lower band is the expected product. The deviation in protein expression level was compensated by normalization of luciferase activity with the coexpressed β -Gal activity. (F) Inhibition of basal transcription from a natural promoter by Daxx. Wild-type Daxx was transfected into HEK293 cells together with either the SF1-tk-luciferase or tk-luciferase reporter. The fold repression of the luciferase activity at increasing concentrations (micrograms) of Daxx is presented.

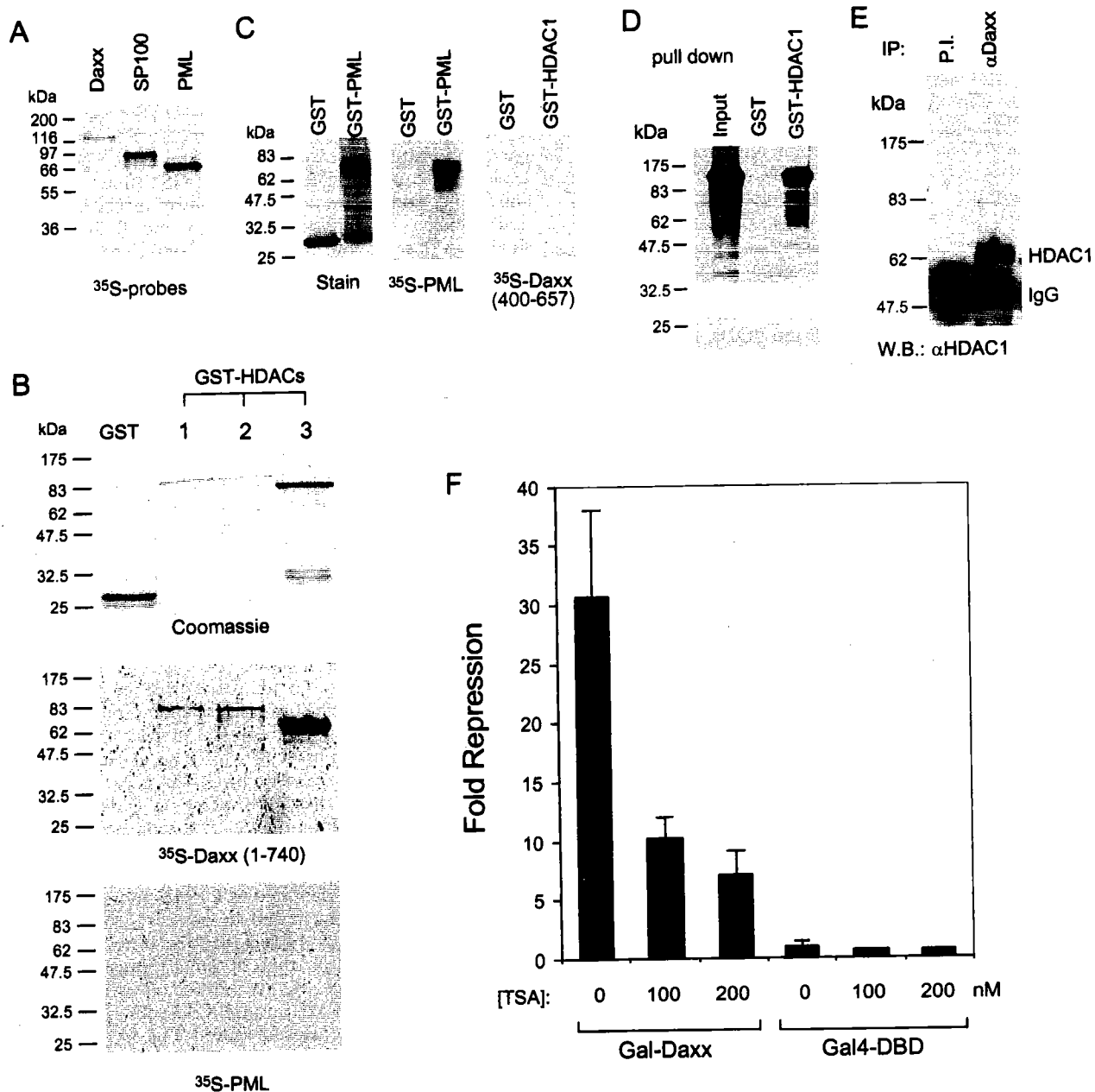


FIG. 4. Interaction of Daxx with HDACs. (A) ^{35}S -labeled protein probes used in the far-Western assays. In vitro-translated [^{35}S]methionine-labeled Daxx, PML, and SP100 were analyzed by SDS-PAGE and detected by autoradiography. (B) Far-Western analysis of interaction between Daxx and HDACs. The top panel shows the Coomassie blue-stained proteins used in the far-Western assay. The middle panel shows the far-Western blot of GST-HDACs with the ^{35}S -labeled Daxx probe. The bottom panel shows the far-Western blot with the ^{35}S -labeled PML probe. (C) PML interacts with GST-PML in the far-Western assay. A positive control showing that PML interacts with GST-PML in the far-Western assay was conducted under conditions identical to those for panel B. A far-Western blot showing that the Daxx mutant (400-657) fails to interact with GST-HDAC1 fusion protein. (D) GST pull-down assay showing interaction between GST-HDAC1 and Daxx. The input ^{35}S -labeled Daxx contains one-third of the lysate used in the pull-down reaction, which was conducted as described in the Materials and Methods. (E) Co-IP of HDAC1 and Daxx. HeLa nuclear extracts were incubated with affinity-purified anti-Daxx antibody or an equal concentration of the preimmune serum. The immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blot by using anti-HDAC1 polyclonal antibodies. (F) TSA reverses transcriptional repression by Gal-Daxx. HEK293 cells were transfected with 250 ng of Gal4-DBD or Gal-Daxx mammalian expression vector together with a Gal4-dependent luciferase reporter. The fold repression by Gal-Daxx at different concentrations of TSA was determined relative to that for the Gal4-DBD alone.

When either the short or long forms of PML-RAR α were cotransfected with Gal-Daxx, the repression activity of Gal-Daxx was unchanged (Fig. 5B). Thus, despite the observation that both PML and PML-RAR α interact with Daxx, only PML can inhibit the ability of Daxx to repress transcription, suggesting a differential role of PML and its oncogenic fusion protein in regulation of Daxx function.

PML recruits Daxx to the POD. To elucidate the mechanism by which PML blocks Daxx-mediated transcriptional repression, immunofluorescence microscopy was used to investigate the subcellular localization of Gal-Daxx upon coexpression of PML. In these experiments, HEK293 cells were transiently transfected with Gal-Daxx in the absence or presence of HA-PML and subsequently stained with the mouse anti-Gal4-DBD and

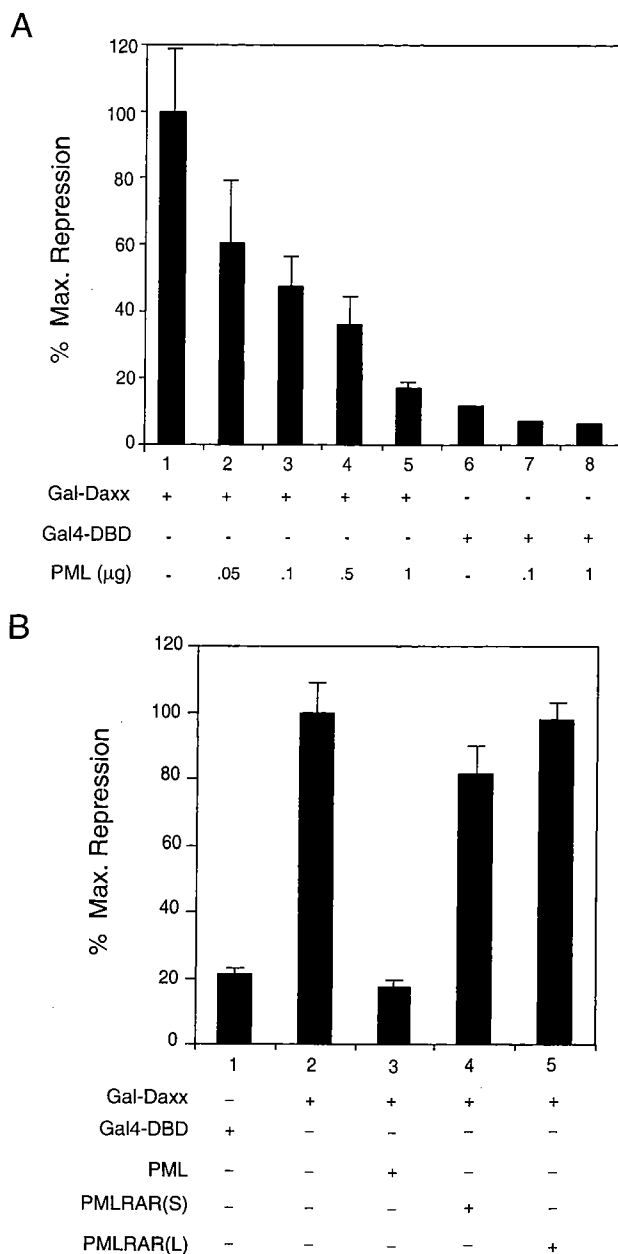


FIG. 5. Inhibition of Daxx-mediated transcriptional repression by PML. (A) PML inhibits Daxx-mediated transcriptional repression. HEK293 cells were transiently transfected with 100 ng of the Gal4-DBD or Gal-Daxx mammalian expression vectors in the absence or presence of the indicated amounts of PML expression vector together with a Gal4-dependent luciferase reporter. Data are presented as the percentage of maximum repression, where Gal-Daxx activity is represented as 100% repression. (B) PML-RAR α has no effect on Daxx-mediated transcriptional repression. HEK293 cells were transiently transfected with 100 ng of the Gal4-DBD or Gal-Daxx mammalian expression vectors in the absence or presence of expression vectors for PML, PML-RAR α (short form), or PML-RAR α (long form), together with a Gal4-dependent luciferase reporter. Data are presented as the percentage of maximum repression, where Gal-Daxx activity is represented as 100% repression.

rabbit anti-HA antibodies (Fig. 6A). When Gal-Daxx was overexpressed alone in HEp2 cells, a fairly diffuse, evenly distributed staining pattern is observed in the nucleus (Fig. 6A, panels a and b). Cotransfection of PML drastically alters the

distribution of Gal-Daxx, for nearly all of the Gal-Daxx protein is recruited to the PODs, even at very high levels of Gal-Daxx expression (Fig. 6A, panels c to f). Examination of the localization of these enlarged PODs indicates that they occupy the loose chromatin regions (Fig. 6Ae and f), similar to the localization of PODs in the absence of PML overexpression. On the contrary, cotransfection of PML does not recruit a Daxx mutant (Gal-Daxx 1-502) lacking the PML-interacting domain to the PODs (Fig. 6A, panels g to j), suggesting the specificity of the assay. The abilities of PML to reverse Daxx-mediated repression and to recruit Daxx to the PODs support the hypothesis that PML may inhibit Daxx repressor function by sequestration of Daxx to the PODs.

PML recruits endogenous Daxx to the PODs. To address whether recruitment of Daxx to the PODs also occurs at the endogenous levels of Daxx, HA-PML was transfected into HEp2 cells alone, and the localization of endogenous Daxx was analyzed by immunofluorescence staining by using anti-Daxx antibodies (Fig. 6B). Double immunostaining with anti-PML antibodies reveals that overexpression of PML leads to accumulation of endogenous Daxx to the PODs, resulting in reduced nucleoplasmic staining (Fig. 6B, panels a to c). Recruitment of endogenous Daxx to the PODs is confirmed with anti-HA antibodies that detect only the transfected HA-PML (Fig. 6B, panels d to f). These data indicate that PML is able to recruit endogenous nucleoplasmic Daxx to the PODs.

PML does not recruit HDAC1 to the PODs. So far we have shown that Daxx interacts with HDACs (Fig. 4) and that PML recruits Daxx to the PODs (Fig. 6). Accordingly, we wished to determine the localization of HDAC and other corepressors, such as SMRT, after PML overexpression. We find that overexpression of PML does not alter the distribution of HDAC1 or SMRT (Fig. 6B, panels g to i), suggesting that PML may segregate Daxx away from the corepressor complex. These observations are consistent with a speculative mechanism by which PML may inhibit transcriptional repression of Daxx via sequestration of Daxx to the PODs.

SUMO-1 modification of PML is required for recruitment of Daxx to the PODs. To determine if SUMO-1 modification of PML may play a role in Daxx interaction, we generated a PML mutant with all three SUMO-1 modification lysine residues replaced with arginines by site-directed mutagenesis, based on a prior study that mapped the modification sites (31). Upon mutation of the three lysine residues of PML, we no longer observe SUMO-1-conjugated forms of PML, even after treatment of the transfected cells with As₂O₃ and coexpression with SUMO-1 (Fig. 7A). This PML Δ SUMO mutant behaves similarly to the wild-type protein in localizing to the PODs and in enlarging the POD structure (Fig. 7B, panels a, d, and g). Interestingly, while this SUMO-1-deficient mutant is capable of localizing to PODs (panels b and c and panels d and e), overaccumulation of the mutant protein in the PODs fails to recruit nucleoplasmic Daxx (Fig. 7B, panels g to i). In contrast, many of the enlarged PODs show reduced staining of Daxx (Fig. 7B, panels g to i), suggesting that accumulation of the unmodified form of PML in the PODs may lead to the disappearance of Daxx in PODs. These data suggest SUMO-1 modification as being the underlying mechanism for the observed interaction and colocalization between Daxx and PML in vivo.

SUMO-1 modification of PML is required for efficient inhibition of Daxx-mediated repression. If our hypothesis that recruitment of Daxx to the POD is inhibitory to its transcriptional repression activity, one would predict that the PML Δ SUMO mutant that fails to recruit Daxx to the PODs will be defective in reversing transcriptional repression by Daxx. As expected, we found that the PML Δ SUMO mutant is less

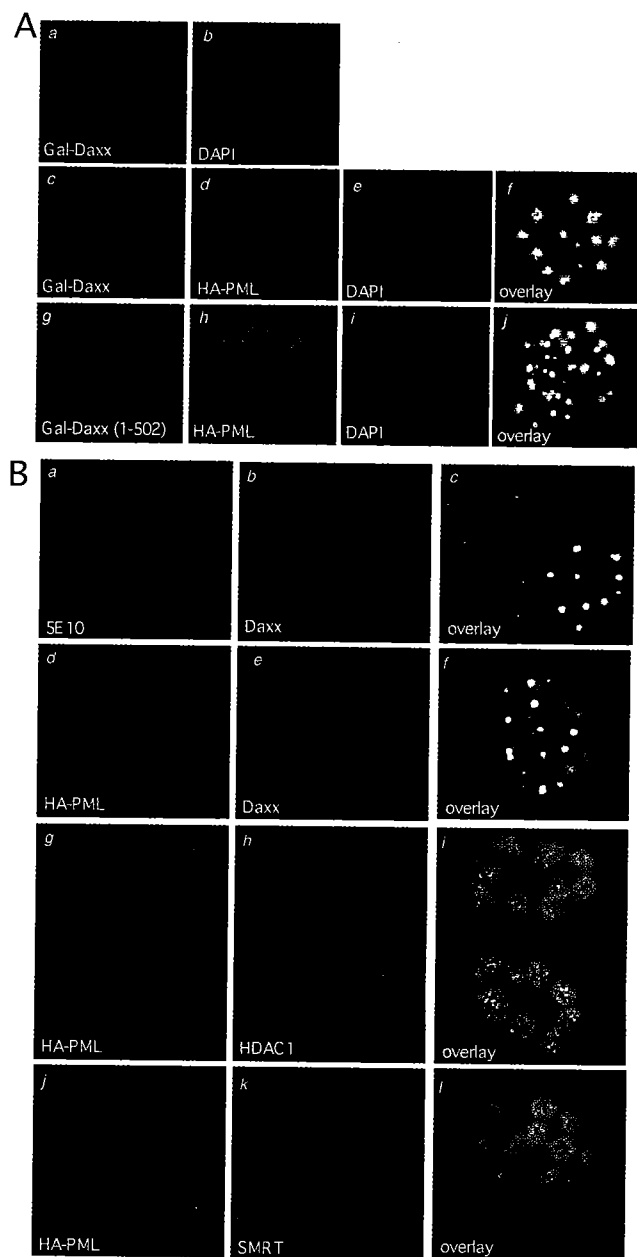


FIG. 6. Recruitment of Daxx to POD domains by overexpression of PML. (A) Overexpression of PML recruits transfected Gal-Daxx into the PODs. Gal-Daxx or Gal-Daxx (1-502) were transiently transfected into Hep2 cells in the absence or presence of HA-tagged PML and subsequently stained with the mouse anti-Gal4-DBD and rabbit anti-HA antibodies. Primary antibodies were detected with rhodamine-conjugated anti-mouse immunoglobulin G and fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G secondary antibodies and analyzed by immunofluorescence microscopy. Panels a and b show diffuse nuclear staining of Gal-Daxx in the absence of PML. Panels c to f show Gal-Daxx and HA-PML colocalization at the PODs. Panels g to j show that HA-PML cannot recruit a Gal-Daxx (1-502) mutant lacking the PML-interacting domain to the PODs. (B) Recruitment of endogenous Daxx but not HDAC1 and SMRT to the PODs. HA-PML was transfected into Hep2 cells, and the localization of endogenous Daxx, HDAC1, and SMRT was analyzed by immunofluorescence microscopy. Panels a to c show colocalization of transfected and endogenous PML with endogenous Daxx by using anti-PML monoclonal 5E10 and anti-Daxx rabbit polyclonal antibodies. Panels d to f show HA-PML and Daxx colocalization by using the anti-HA monoclonal and anti-Daxx polyclonal antibodies. Panels g to l demonstrate that HA-PML does not recruit HDAC1 or SMRT to the PODs by using anti-HA, anti-HDAC1, or anti-SMRT antibodies. Yellow signals in the overlay images indicate colocalization.

effective in reversing transcriptional repression by Daxx in transient transfection (Fig. 7C). Furthermore, we found that the wild-type PML is incapable of reversing transcriptional repression by Gal-HDAC1 and Gal-SMRTe (Fig. 7C). These data correlate with immunofluorescence studies demonstrating PML recruitment of Daxx, but not HDAC or SMRT, to the POD, where it presumably is unable to repress transcription.

Daxx is associated with condensed chromatin in the absence of PML. To provide further evidence that the demonstrated functional interactions between Daxx and PML may be physiologically relevant, we screened several cell lines to find a cell type that may display abnormal localization of Daxx and/or PML. We identified the embryonic carcinoma NT2 cell line; upon staining with the anti-PML antibody, it is evident that only a subset of these cells express PML and thus contain PODs (Fig. 7D). In these cells, PML and Daxx colocalize in the PODs (panels a to d). However, in cells lacking detectable PODs, Daxx forms aggregates around the condensed chromatin (Fig. 7D, panels e to h). Therefore, the localization and thus the function of Daxx may depend on the level of PML in the cell. At low PML levels, Daxx is concentrated at condensed chromatin, where it may repress transcription. When PML levels are higher, it is able to recruit Daxx away from condensed chromatin to the PODs, where Daxx no longer represses basal transcription.

DISCUSSION

In the present study, we have identified Daxx as a PML-interacting protein and characterized the functional interaction between Daxx and PML. We find a majority of Daxx in the nucleus of HeLa and Hep2 cells where it colocalizes with PML in the PODs. In the NB4 APL cell line, Daxx is distributed in the microparticulate structures that contain the PML-RAR α oncoprotein (17). The repressor function of Daxx is observed upon tethering it to a reporter gene via a heterologous DNA binding domain, as well as from a reporter containing a natural SF1-like promoter element. The mechanism by which Daxx represses basal transcription is found as involving histone deacetylation, for Daxx interacts with HDACs in vitro and in vivo and the histone deacetylase inhibitor, TSA, blocks the repressor activity. Coexpression of PML reverses the transcriptional repression by Daxx, which, in turn, correlates with the recruitment of Daxx to the PODs. In addition, we show that SUMO-1 modification of PML is required for both recruitment of Daxx to the PODs and efficient inhibition of Daxx-mediated repression. The physiological role of Daxx in transcriptional repression is further supported by the observation that Daxx associates with condensed chromatin in cells that lack PML. Together, these data establish novel roles for Daxx, as a transcriptional repressor, and for PML, as a protein that can potentially regulate the repressor function of Daxx.

Consistent with our findings, Daxx has recently been identified as an inhibitor of transcriptional activation by Pax3, a member of the homeodomain family of transcription factors (28). Thus, Daxx not only is able to repress basal transcription, as suggested from our data, but can also inhibit transcriptional activation via interactions with DNA-binding transcription factors. While the exact mechanism of the inhibition of Pax3 transactivation by Daxx is unclear, our data elucidate the mechanism of Daxx-mediated repression of basal transcription as involving histone deacetylation. We observe Daxx localization to condensed chromatin in NT2 cells that lack detectable PML. Condensed chromatin is considered as a site of transcriptional repression that also includes transcriptionally silent centromeric heterochromatin. Other POD-associated pro-

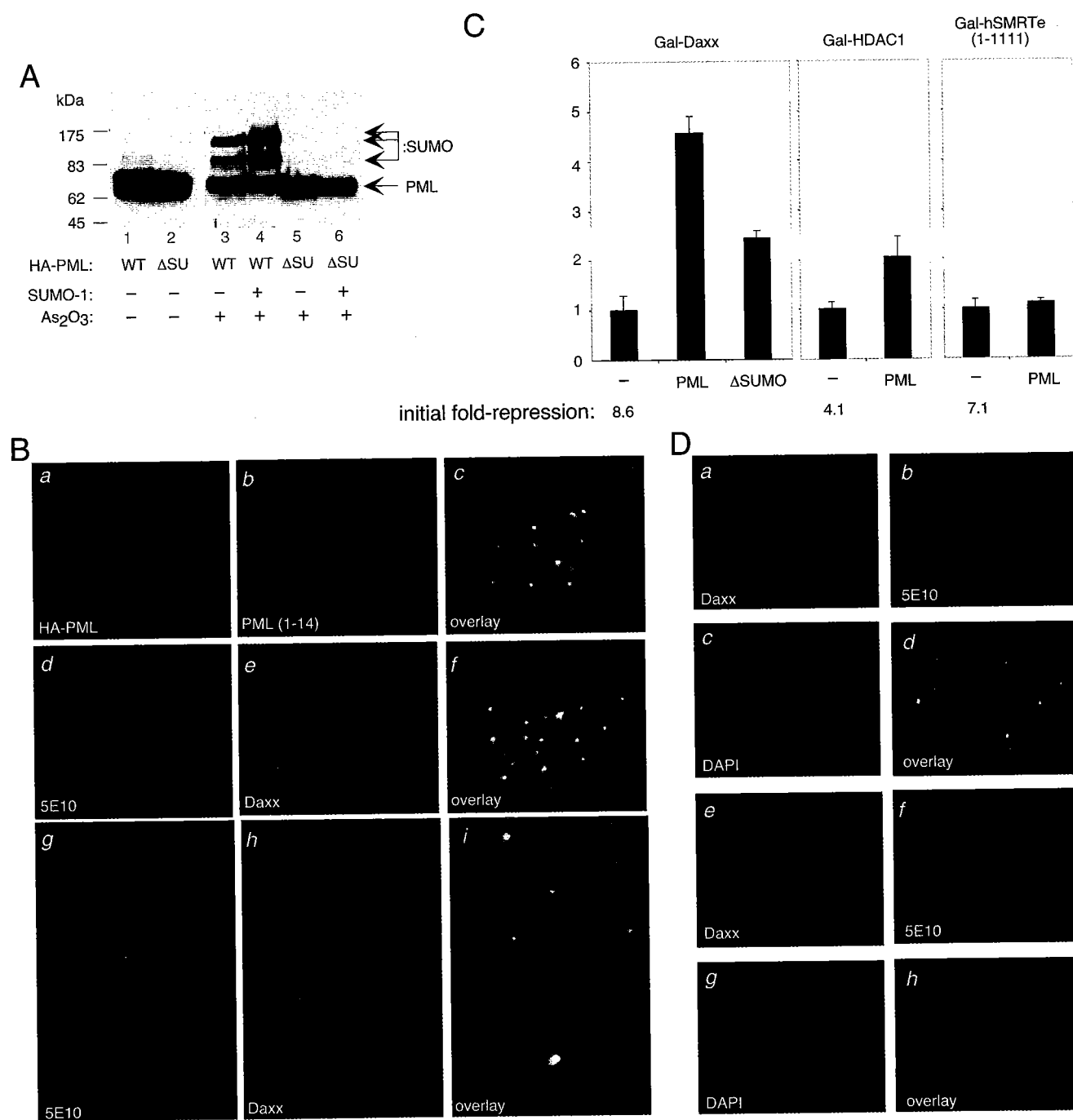


FIG. 7. SUMO-1 modification of PML is required for sequestration of Daxx to the POD and inhibition of Daxx-mediated transcriptional repression. (A) The PML Δ SUMO (Δ SU) mutant lacks SUMO-1 modification. This mutant was created by replacing all three lysines at residues 65, 160, and 490 with arginines. The wild-type (WT) PML and the Δ SUMO mutant were transfected into HEK293 cells alone or in combination with a SUMO-1 expression vector. Cells were treated with 1 μ M arsenic trioxide (As_2O_3) for 6 h where indicated. The total cell lysates were analyzed by Western blotting by using anti-HA monoclonal antibodies. The upper bands in the wild-type proteins represent SUMO-1 conjugated forms of PML. (B) The PML Δ SUMO mutant localizes to the PODs but fails to recruit Daxx. The HA-PML Δ SUMO mutant was transfected into HEK293 cells and analyzed by immunofluorescence microscopy to detect localization of the transfected mutant protein and the distribution of endogenous Daxx. Panels a to c show localization of the transfected HA-PML Δ SUMO mutant protein (detected by a HA antibody) in the PODs that were revealed by a PML polyclonal antibody (1-14). Panels d to f show that the enlarged PODs in the HA-PML Δ SUMO mutant transfected cells do not result in prominent recruitment of Daxx to the PODs. Panels g to i show that many enlarged PODs containing PML- Δ SUMO mutant have little or no Daxx protein. The 5E10 antibodies also detect PODs in untransfected cells that show smaller structures colocalized with Daxx foci. (C) The PML Δ SUMO mutant is deficient in reversing transcriptional repression by Daxx. The transfection was conducted in HEK293 cells, and the initial fold repression mediated by the Gal4-DBD fusion proteins is as indicated at the bottom. The y axis indicates the fold reversal of repression. The wild-type PML does not reverse transcriptional repression mediated by HDAC1 or SMRte. (D) Association of Daxx with condensed chromatin in cells that lack PODs. The human neuronal NT2 stem cells were analyzed by double immunofluorescence staining with anti-Daxx polyclonal and anti-PML 5E10 monoclonal antibodies. The NT2 cells display heterogeneous staining for PML. In cells that contain normal PML nuclear bodies (panels a to d), Daxx appears normal and shows complete colocalization with PML. In contrast, cells that contain only two or fewer PML nuclear dots show aggregated Daxx surrounding the condensed chromatin areas stained with DAPI (panels e to h).

teins, such as SP100, have been demonstrated to interact with heterochromatin protein 1 (HP1) and also colocalize with centromeric chromatin (10, 54). Consistent with this idea, Daxx has been shown to interact with CENP-C in a yeast two-hybrid assay and partially to colocalize with interphase centromeres (50). Also, Daxx has been shown to interact with DNA methyltransferase 1, which plays a role in gene silencing (43).

Previous studies have implicated the PODs as sites of transcriptional activation. For example, PML has been demonstrated to interact with the transcription coactivator CBP and recruit CBP to the PODs (15, 35). Furthermore, PML can enhance the transactivation functions of both CBP and members of the nuclear receptor superfamily (15). PML also induces genes of the major histocompatibility complex, while PML^{-/-} mice display reduced transactivation responses to atRA (64, 69). Finally, the transcriptional activator Sp140 (5, 6) and nascent RNA (35) have been found in at least a subset of PODs. Our findings that Daxx represses basal transcription and PML inhibits such repressor activity suggest a new role for the POD structure in gene regulation. The POD may enhance transcription of target genes not only through recruitment of activators but also through the inactivation of repressors such as Daxx via recruitment by PML. Because other transcriptional repressors, such as PLZF, pRB, and Sp100, have also been found in the PODs, it will be interesting to determine if PML can regulate the repressor activities of these proteins as well.

Our observations that PML-RAR α can interact with Daxx but not inhibit transcriptional repression by Daxx suggest a potential role for Daxx in acute promyelocytic leukemia. Support for this hypothesis is evident in our finding that Daxx, PML-RAR α , and PML colocalize at diffusely distributed microparticulate structures in nucleus of the APL NB4 cells. The PML-RAR α fusion protein disrupts the POD structure in these cells and, through its interaction with Daxx, may direct Daxx to the microparticulate structures, where it is capable of repressing gene expression. PML-RAR α itself is a potent transcriptional repressor, which acts via the recruitment of the corepressors SMRT, N-CoR, and HDAC1 (40). The POD structure is reorganized upon treatment of these cells with atRA or arsenic trioxide, leading to the degradation of the PML-RAR α fusion protein and colocalization of Daxx and PML in the PODs (47). Therefore, Daxx inactivation through localization to the PODs may be critical to the differentiation of normal hematopoietic cells. Since expression of the PML-RAR α fusion protein disrupts the integrity of the PODs, Daxx may act as a constitutive repressor in the APL cells, which along with the repressor function of PML-RAR α , may block expression of specific genes that are critical for cell differentiation and culminate in the subsequent APL pathology.

Daxx was initially identified as a Fas-binding protein that promoted Fas-mediated apoptosis via activation of the JNK kinase cascade pathway (12, 68). Interestingly, PML has also been found to be involved in apoptosis triggered by Fas, tumor necrosis factor alpha, and type I and II interferons, possibly by recruitment of the death effector Bax and cdk inhibitor p21 (37, 51, 64). In contrast, expression of PML-RAR α prevents apoptosis in response to these signals (51). Our findings, together with these reports, suggest that the regulation of Daxx repressor function by PML may also be important in programmed cell death. Consistent with this possibility, several transcriptional repressors are known to play a role in apoptosis. For example, the adenovirus E1B and the cellular Bcl-2 oncoprotein block p53-mediated apoptosis by inhibiting transcriptional repression by p53, suggesting that p53 induces apoptosis via transcriptional repression (52, 56). In the case of Daxx, PML may recruit it to the PODs, where it is inactivated,

thus allowing the expression of certain genes required for apoptosis. Conversely, PML-RAR α might inhibit apoptosis in APL cells through disruption of the PODs, thereby promoting enhanced or constitutive repression of these target genes by Daxx and the PML-RAR α fusion protein itself, which leads to the APL phenotype. Retinoic acid treatment would stimulate degradation of PML-RAR α and restoration of the POD structure (17, 47, 65). This would allow Daxx to be inactivated through sequestration to the PODs and allow apoptosis to proceed and would eventually lead to remission of the APL phenotype. Because PML can shuttle between the nucleus and cytoplasm (59, 60), it is possible that Daxx may be brought along with PML to regulate cytoplasmic events relevant to Fas-mediated apoptosis. However, a recent study reports that the loss of Daxx leads to extensive apoptosis in early mouse development (43), a result seemingly opposite to other findings concerning the function of Daxx in apoptosis (12, 13, 68). Therefore, the precise role of Daxx in apoptosis remains to be further elucidated.

Our data provide strong evidence for the roles of PML and the PODs in regulating the function of Daxx as a transcriptional repressor. Daxx and PML interact *in vivo* and colocalize in the PODs. Overexpression of PML recruits Daxx to the PODs, which correlates with a complete inhibition of transcriptional repression by Daxx. Although the detailed mechanism of this inhibition of Daxx by PML remains to be determined, our data provide several possibilities. First, PML might inactivate Daxx by transporting it to the PODs and separating it from HDAC and putative target genes. In response to certain stimuli such as interferon, PML levels increase in the PODs, which, via competition for Daxx binding or conformational change of Daxx upon PML binding, might result in the dissociation of Daxx from HDAC and recruitment of Daxx, but not HDAC, to the PODs. Confinement of Daxx in the PODs would thus block access to target genes, whose expression level would then increase to at least the basal level in the absence of Daxx repression. Our findings that PML overexpression results in increased Daxx levels in the PODs, while having no effect on HDAC1 distribution or repression by HDAC1, support this possibility. Alternatively, the increased PML levels may dissociate HDAC from Daxx and recruit both Daxx and its putative target genes, but not HDAC, to the PODs. Because Daxx requires HDAC and histone deacetylation for its repressor activity, the target genes may be expressed in the absence of HDAC. The presence of transcriptional activators in the PODs would facilitate transcription of target genes. With either possibility, it is evident that the POD is involved in maintaining the balance of Daxx function, depending on the PML level. At normal, physiological levels of PML, Daxx might repress transcription at areas of condensed chromatin. However, with increased PML expression, more Daxx is recruited to the PODs, thus reducing its overall repression activity. Although the precise mechanism of the inhibition of Daxx repression by the PODs awaits further investigation, our data clearly reveal a novel connection between Daxx and PML in regulating transcriptional repression that may play a critical role in acute promyelocytic leukemia and apoptosis.

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Characterization of Receptor Interaction and Transcriptional Repression by the Corepressor SMRT

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SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) and **N-CoR** (nuclear receptor corepressor) are two related transcriptional corepressors that contain separable domains capable of interacting with unliganded nuclear receptors and repressing basal transcription. To decipher the mechanisms of receptor interaction and transcriptional repression by **SMRT/N-CoR**, we have characterized protein-protein interacting surfaces between **SMRT** and nuclear receptors and defined transcriptional repression domains of both **SMRT** and **N-CoR**. Deletional analysis reveals two individual nuclear receptor domains necessary for stable association with **SMRT** and a C-terminal helix essential for corepressor dissociation. Coordinately, two **SMRT** domains are found to interact independently with the receptors. Functional analysis reveals that **SMRT** contains two distinct repression domains, and the corresponding regions in **N-CoR** also repress basal transcription. Both repression domains in **SMRT** and **N-CoR** interact weakly with **mSin3A**, which in turn associates with a histone deacetylase **HDAC1** in a mammalian two-hybrid assay. Far-Western analysis demonstrates a direct protein-protein interaction between two **N-CoR** repression domains with **mSin3A**. Finally we demonstrate that overexpression of full-length **SMRT** further represses basal transcription from natural promoters. Together, these results support a role of **SMRT/N-CoR** in corepression through the utilization of multiple mechanisms for receptor interactions and transcriptional repression. (*Molecular Endocrinology* 11: 2025-2037, 1997)

INTRODUCTION

Transcriptional regulation by steroid/thyroid hormones and retinoids is a critical component in controlling many aspects of animal development, reproduction,

and metabolism (1-4). The functions of these hormones are mediated by intracellular receptors, which comprise a large superfamily of ligand-dependent transcription factors (1). It has been established that both retinoic acid receptors (**RARs**) and thyroid hormone receptors (**TRs**) function via formation of heterodimeric complexes with retinoid X receptors (**RXRs**) (5, 6). Once bound to a DNA response element, the heterodimer responds to ligand through the C-terminal ligand-binding domain (**LBD**), which is known to mediate not only hormone binding but also receptor dimerization, transcriptional activation, and repression (7, 8).

Both **TR** and **RAR** can function as transcriptional repressors in the absence of ligands and potent activators upon binding of ligands (7). DNA-binding assays and functional analysis have demonstrated that the repressor activities of unliganded receptors depend on DNA response elements, as well as on the intact **LBD** of the receptors (7, 9, 10). *In vivo*, the **TR/RXR** heterodimer binds to DNA in the context of chromatin, and nucleosome assembly enhances the transcriptional silencing effect (11). Importantly, the oncogenic activity of **v-erbA**, a mutated form of **TR**, is directly linked to transcriptional repression (12, 13). In addition, deletion of the activation domain of **RAR** converts it into a potent transcriptional repressor, and this mutation was shown to cause defects in cellular differentiation and development (14-16). Therefore, transcriptional repression by unliganded nuclear receptors appears to play an important role in regulating cell growth and differentiation.

Hormone binding is thought to induce conformational changes that lead to ligand-dependent transformation of the receptors from repressors to activators (1). The C terminus of **TR**, about 20 amino acids, constitutes the 12th amphipathic helix (helix 12) of the **LBD** (17-19), which functions as a ligand-dependent activation core domain known as the **AF2-AD**, τ C, or τ 4 domain (8, 20-22). Comparison of the **LBD** structures of the unliganded (19) and liganded receptors (17, 18) reveals a striking difference in the relative position of the helix 12/**AF2-AD** domain. This posi-

tional shift is thought to play an important role in receptor activation, allowing the liganded receptors to displace corepressors (8, 23–25) and to interact with coactivators (see reviews in Refs. 26–28).

SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) and N-CoR (nuclear receptor corepressor) are two related transcriptional corepressors (24, 25) that are distinct from other proteins (29). They were shown to utilize the C-terminal domain for interaction with unliganded receptors (30–33), and the N-terminal domain for transcriptional repression (25, 30). In this study, we investigate mechanisms of protein-protein interactions between SMRT and nuclear receptors and analyze the modes of repression mediated by SMRT/N-CoR. To do this, we define the interacting surfaces between SMRT and nuclear receptors in binding and functional assays. Next, we compare transcriptional repression mediated by SMRT and N-CoR using transient transfection assays in mammalian cells. Evidence is presented that SMRT and N-CoR interact with additional corepressors, and that histone deacetylation plays a role in SMRT/N-CoR-mediated repression.

RESULTS

Two Receptor Domains Are Essential for Interaction with SMRT

Deletion mutants in the carboxyl and amino termini of TR and RAR were used to analyze the contribution of different regions in the receptors for protein-protein interaction with SMRT. Figure 1A shows the domain structure of TR and the relative position of individual helices in the LBD as determined by x-ray crystallography (17, 18). The sequence at the C terminus region around helices 11 and 12 is also shown for both TR and RAR. [³⁵S]Methionine-labeled TR or RAR deletion mutants were hybridized to glutathione S-transferase (GST)-SMRT and GST-RXR in far-Western analyses in the absence of hormone (Fig. 1B). The relative strengths of these interactions are summarized in Fig. 1C.

Full-length TR (1–456) associates well with both SMRT and RXR, and the interaction with SMRT can be drastically reduced upon hormone treatment. A residual weak interaction was observed in the presence of ligand, consistent with previous observations (24, 30). Carboxyl-terminal truncation at residue 441, which deletes helix 12, results in a mutant that interacts normally with RXR but that exhibits enhanced interaction with SMRT. Further truncation at residue 423, which removes part of helix 11, reduces the interaction with SMRT back to a level similar to that of wild type TR. In contrast, this deletion markedly reduces interaction with RXR. Further deletions that remove additional helices (helices 8, 9, and 10) result in barely detectable interaction with SMRT and no interaction with RXR. These results suggest that helix

12 inhibits SMRT association while helix 11 might promote the association.

Amino-terminal truncation of TR at residue 173, which removes the DNA-binding domain (DBD), does not affect the interaction with either SMRT or RXR. Further N-terminal deletion to residue 260, which removes the first and second helices of the TR LBD, markedly impairs SMRT association. No interaction with RXR by this mutant was detectable. Similarly, C-terminal deletion of helix 12 from RAR (1–403) also increases interaction with SMRT as compared with that of wild type RAR (1–462). Further deletion to residue 395, which removes part of helix 11, diminishes the enhanced interaction to a level comparable with that of full-length RAR, and ligand has little effect on the interaction. Together, these results identify two distinct interacting domains at the N-terminal hinge and C-terminal helix 11 regions of the receptor LBD that might act synergistically to promote interaction with SMRT. We find that the other two RAR isoforms, β and γ , also interact with SMRT in a ligand-reversible manner, although the interactions observed are weaker compared with that with RAR α (Fig. 1D). The interactions of both RAR β and RAR γ with RXR were not affected by ligand treatment.

Interaction of Helix 12/AF2-AD Deletion Mutants with SMRT in Yeast

To further understand the role of helix 12/AF2-AD in interaction with SMRT, we analyzed interactions between AF2-AD deletion mutants of RAR and RXR with C-terminal receptor-interacting domain of SMRT in a yeast two-hybrid system (Fig. 2). The RAR LBD alone is sufficient to interact with SMRT in a ligand-reversible manner (Fig. 2A, column 3), but the resulting activity is much weaker compared with that of full-length RAR (column 9). Similar to the far-Western results, SMRT and full-length RAR retain some interaction, even after treatment of the yeast cells with a saturating amount of ligand. It is unclear whether this observation reflects an association between liganded receptors and SMRT or the existence of a small percent of unliganded receptors after ligand treatment. Deletion of the AF2-AD domain results in a RAR mutant that stimulates gene expression in response to hormone treatment in yeast (columns 4 and 10), as opposed to the dominant negative activity of this mutant observed in mammalian cells (14). The ligand-dependent activation of RAR403 is more obvious in the context of full-length receptor (column 10). A similar effect has been shown in v-erbA, which normally acts as a constitutive repressor in mammalian cells, but as a ligand-dependent activator in yeast (34). Cotransformation of the RAR403 mutants with a Gal4 activation domain-SMRT fusion (Gal4 AD-SMRT) strongly induces β -galactosidase expression, even in the absence of hormone (columns 5 and 11). Furthermore, in contrast to the hormone-dependent dissociation seen with full-length RAR, hormone treatment does not interrupt

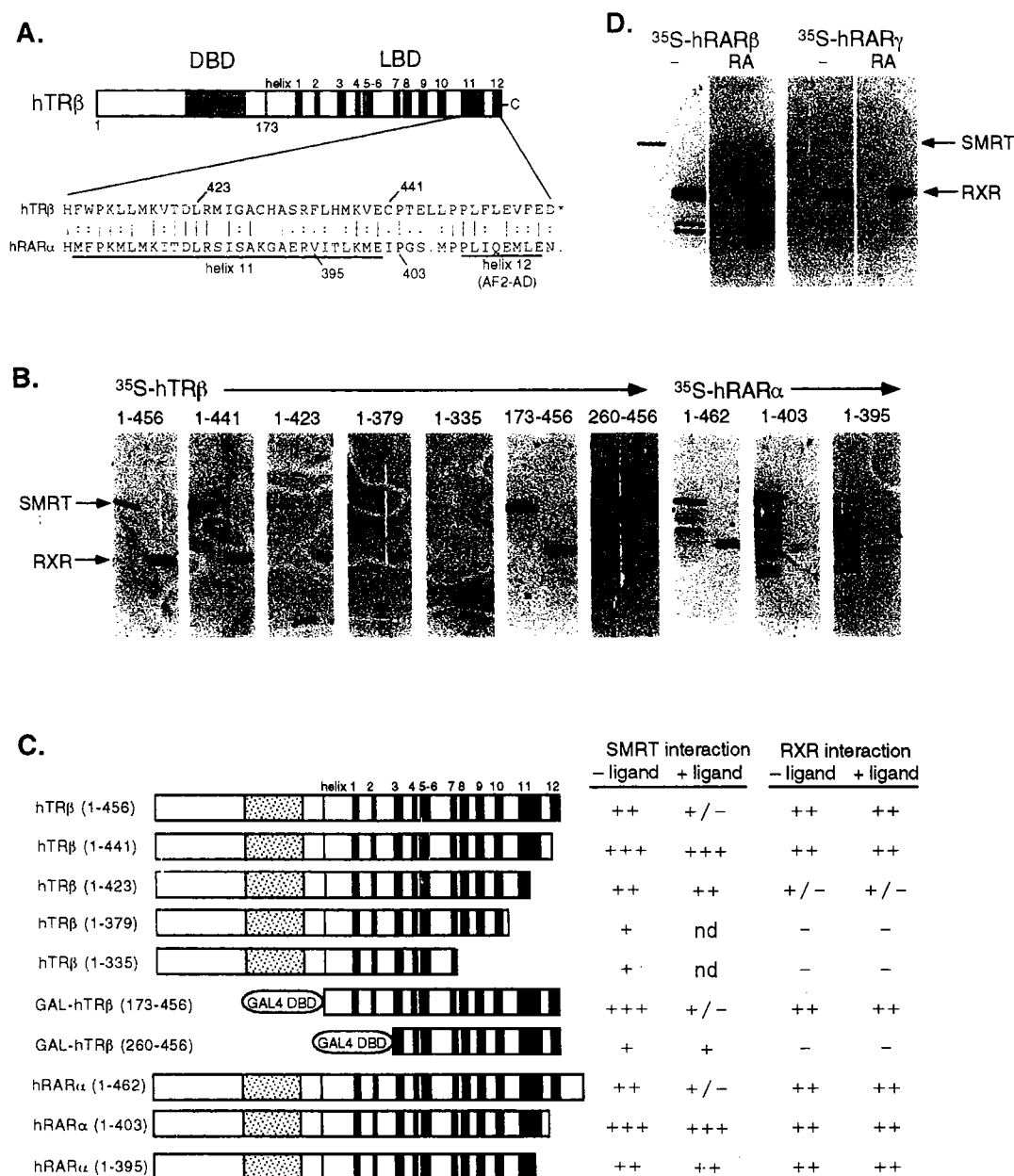


Fig. 1. Two Receptor Domains Interact with SMRT

A, Domain structure of human TR β and the sequences of the C-terminal helix 11 and 12 (AF2-AD) region of TR and RAR. The relative positions of individual helices determined by x-ray crystallography (18) are also indicated. B, Protein-protein interactions between receptors and SMRT or RXR in far-Western analyses. The full-length TR and RAR and their deletion derivatives were translated *in vitro* and labeled by [35 S]methionine. All these deletion mutants expressed similar amounts of proteins as analyzed by SDS-PAGE and autoradiography (not shown). The position of GST-C-SMRT (SMRT) and GST-RXR (RXR) fusion proteins are as indicated (arrows). Please note that GST-RXR appeared as a doublet in our extract. C, Summary of relative levels of interactions between receptor mutants and SMRT or RXR. The relative levels of interactions were scored from background level (-) to strong (+++). nd, Not done. D, Human RAR β and RAR γ interact with SMRT in a ligand-reversible manner in far-Western blots. -, vehicle only; RA, 1 μ M of all-*trans*-retinoic acid.

these interactions. Similarly, the Gal4 DBD-SMRT fusion interacts strongly with the Gal4 AD-RAR403 mutants in a ligand-insensitive manner (columns 6 and 12). These results are consistent with the enhanced interaction observed *in vitro* and indicate that the

AF2-AD domain may act as a negative regulatory element, controlling hormone-sensitive interaction between SMRT and nuclear receptors.

The effect of AF2-AD deletion in RXR on association with SMRT was also analyzed in the two-hybrid sys-

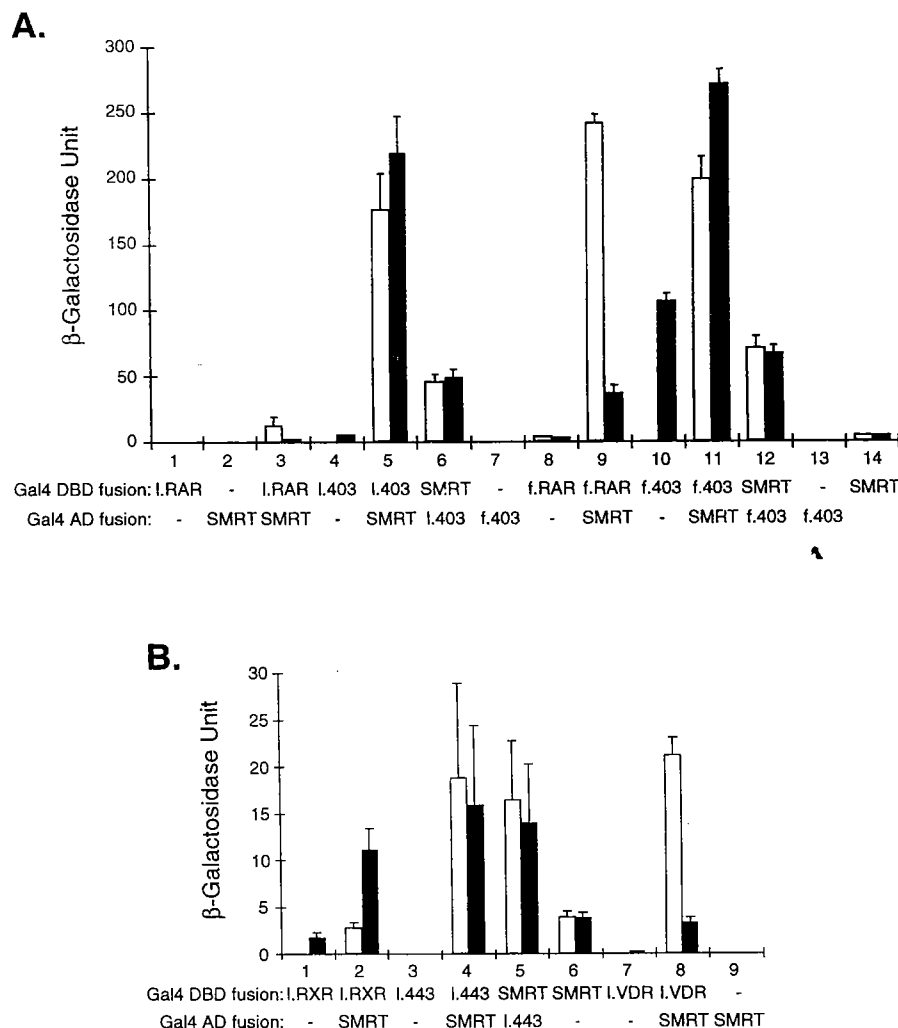


Fig. 2. Two-hybrid Interactions between SMRT and Helix 12/AF2-AD Deletion Mutants of Nuclear Receptors

A. Interaction between RAR403 and C-terminal domain of SMRT in yeast two-hybrid system. The indicated Gal4 AD and Gal4 DBD fusion constructs were cotransformed into yeast Y190 cells, and the resulting β -galactosidase activities were determined from three independent colonies. The β -galactosidase activities were determined in the absence (*open bars*) or presence (*closed bars*) of $1 \mu\text{M}$ of all-*trans*-RA. l, Ligand binding domain; f, full length; 403, RAR403 mutant with C-terminal truncation at residue 403. **B.** Interaction of SMRT with RXR443 and VDR in the absence of hormone (*open bars*) or presence (*closed bars*) of $1 \mu\text{M}$ 9-*cis*-RA (for RXR) or 100 nM 1,25 dihydroxyvitamin D_3 (for VDR). 443, RXR443 mutant with C-terminal truncation at residue 443.

tem (Fig. 2B). Ligand treatment weakly activates the Gal4 DBD-RXR LBD fusion (column 1), while cotransformation with Gal4 AD-SMRT enhances reporter gene expression (column 2), suggesting that SMRT can interact with RXR in either absence or presence of ligand. Truncation at residue 443 enhances the association between RXR and SMRT, and treatment with ligand does not alter this interaction (columns 4 and 5). These results suggest that SMRT can interact with RXR and that the AF2-AD domain of RXR also acts negatively in SMRT association. Furthermore, we observed a significant interaction between vitamin D_3 receptor (VDR) and SMRT in the absence of hormone, and treatment with ligand reduces the interaction (column 8). This result is consistent with the recent finding that VDR also contains intrinsic transcriptional repres-

sion activity (35), suggesting that SMRT might mediate transcriptional repression by VDR.

Two SMRT Domains Mediate Differential Interactions with Nuclear Receptors

The finding that two regions of TR are essential for protein-protein interaction with SMRT suggests that SMRT might also contain duplicated receptor-interacting domains. Several deletion mutants of SMRT were used to test this possibility in a far-Western blot, and the results are summarized in Fig. 3A. The GST fusions of these SMRT mutants were overexpressed, and the purified proteins (Fig. 3B, lanes 1 and 2) or crude extracts (lanes 3, 4, and 5) were analyzed for interaction with ^{35}S -labeled RAR and TR. SMRT(981-

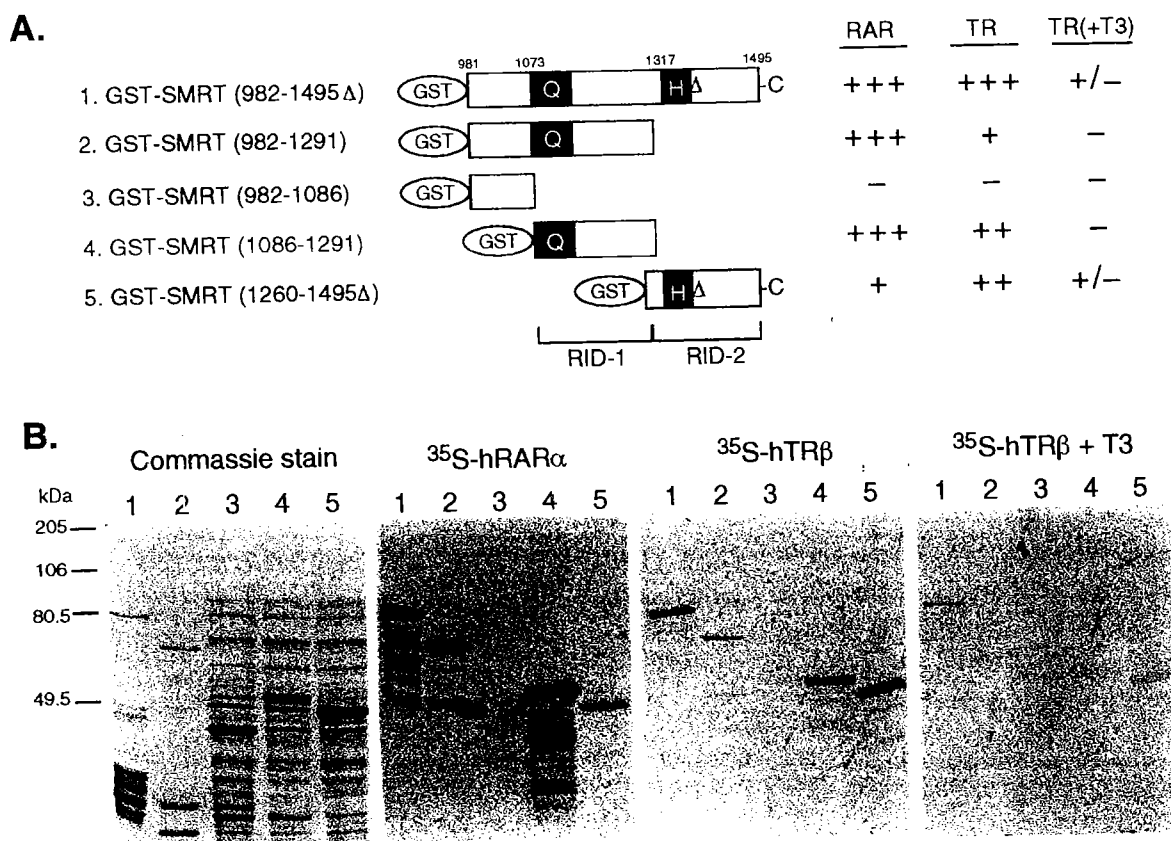


Fig. 3. Two SMRT Domains Interact with the Receptors

A, Summary of SMRT deletion mutants used in this experiment and their relative levels of interaction with nuclear receptors in far-Western analyses shown in panel B. The amino acids encoded by the SMRT mutants are shown in *parentheses*. Bound-RAR and TR were detected by autoradiography, and the relative levels of interaction were scored from background level (-) to strong (+++). The column numbers in each panel correspond to constructs shown in panel A. Partially purified GST fusion proteins were used in lanes 1 and 2 and total cell extracts were used in lanes 3, 4, and 5. RID, Receptor interacting domain. + T₃, Plus 1 μM T₃; Q, glutamine-rich domain; H, putative helical region; Δ, an internal deletion between amino acids 1330 and 1375 resulting from alternative splicing.

1495Δ) interacts equally well with both RAR and TR in the absence of ligands. RAR, but not TR, also interacts with degradation products of SMRT(981-1495Δ). Similarly, several fast migrating products of SMRT(1086-1291) also interact well with RAR, but not with TR (lane 4). These results indicate that RAR and TR may interact differently with SMRT. Consistent with this speculation, we find that SMRT(982-1291) (lane 2) as well as SMRT(1086-1291) interact more strongly with RAR than with TR. In contrast, the C-terminal fragment (1260-1495Δ) interacts better with TR than with RAR (lanes 5). All these interactions were found to be sensitive to hormone treatment (Fig. 3B and data not shown). Together, these results identify two independent receptor interacting domains (RID-1 and RID-2) of SMRT that appear to display different affinities to TR and RAR.

Two SMRT Repression Domains

In addition to the C-terminal receptor interacting domains, SMRT/N-CoR proteins also contain strong

transcriptional repression activity at their N-terminal regions. To define the minimal region needed for repression by SMRT, serial SMRT deletion mutants were generated, and their repression activities were analyzed using transient transfection (Fig. 4A). Consistent with previous observations, "full-length as well as N-SMRT (amino acids 1-981) repress basal transcription strongly and in a dose-dependent fashion (rows 2 and 3), while C-SMRT (amino acids 982-1495Δ) exhibits minimal repression (row 4) compared with Gal4 DBD alone (row 1). Further deletion from the C terminus of N-SMRT reveals that amino acids 743 to 981 are not necessary for repression (row 5), while deletion to residue 475 reduces the repression effect about 2-fold (row 6). These results suggest that amino acids 475 to 981 may contribute in part to SMRT repression. Further C-terminal deletion to residue 337 drastically interferes with repression (row 7), indicating that the N-terminal boundary of this SMRT repression domain-1 (SRD-1) is located between amino acids 337

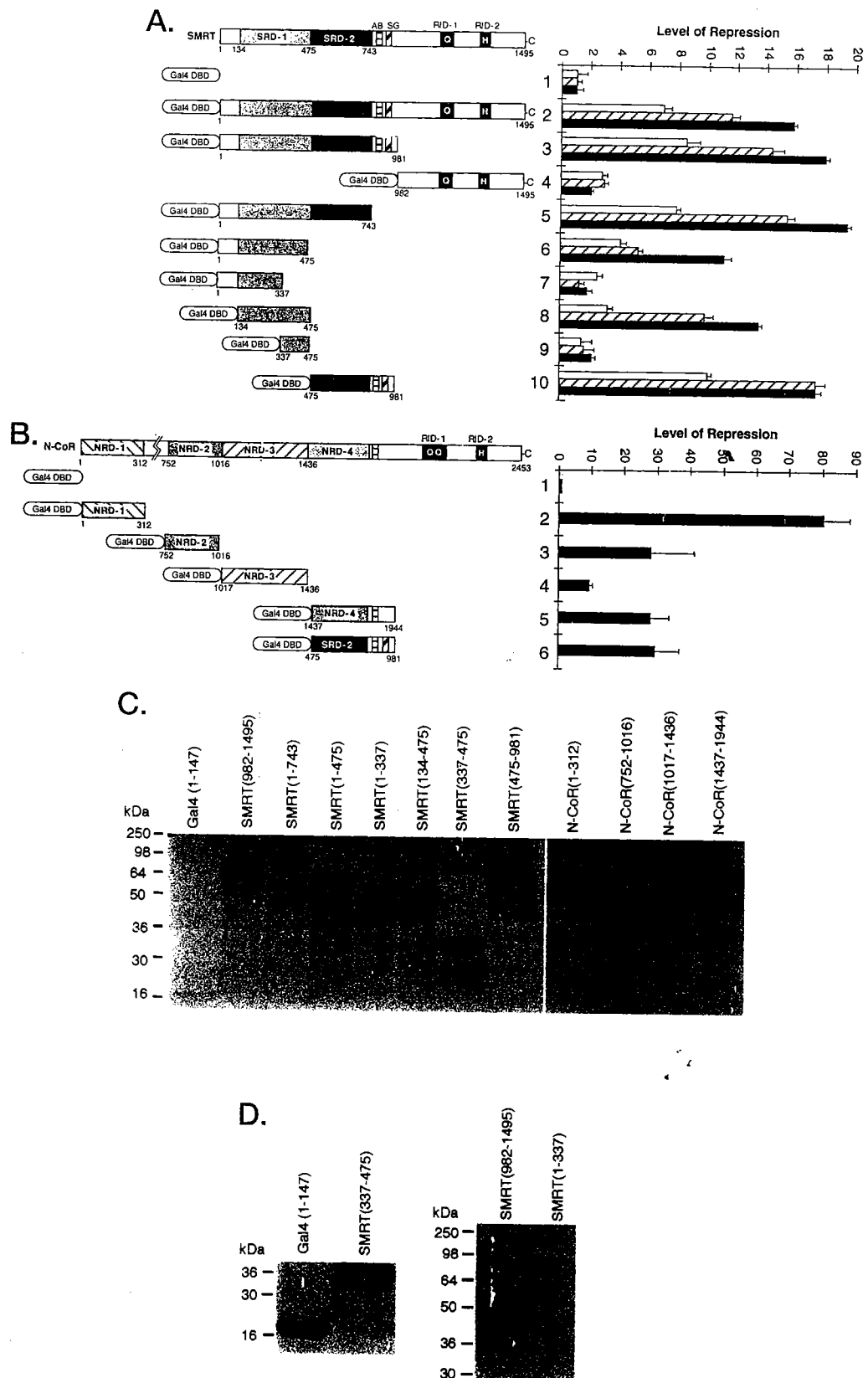


Fig. 4. Multiple Transcriptional Repression Domains

A, Deletion mapping of the repression domains of SMRT. The transcriptional repression activities were analyzed by transient transfection in CV-1 cells. The relative levels of repression were determined from an average of three independent transfections

and 475. Truncation from the N terminus reveals that amino acids 1–134 are dispensable for repression by SRD-1 (row 8), while further deletion to residue 337 abolishes repression (row 9), indicating that the C-terminal boundary of the SRD-1 is within amino acids 134–337. When the SMRT fragment between amino acids 475 and 981 was tested for repression, we found that this fragment also strongly repressed basal transcription (row 10). Together with the observation that amino acids 743–981 are not important for repression, these results may define amino acids 475–743 as a second, independent SMRT repression domain (SRD-2).

Sequence comparison between SMRT and N-CoR reveals that they share about 45% identity within both SRD-1 and SRD-2, suggesting potential functional conservation. Therefore, we tested whether the two SRD corresponding regions of N-CoR also contain repression activities. Consistent with a previous observation (25), amino acids 1–312 and 752–1016 of N-CoR exhibit strong repression activities (Fig. 4B, rows 2 and 3), and the two N-CoR domains corresponding to SRD-1 and SRD-2 also yield 10- to 30-fold repression (rows 4 and 5), similar to the repression effects observed by SRD-1 and SRD-2. These two additional N-CoR repression domains are termed N-CoR repression domain 3 and 4 (NRD-3 and NRD-4), and the two N-terminal repression domains are called NRD-1 and NRD-2. Together, these results indicate that both SMRT and N-CoR contain multiple, independent transcriptional repression domains.

To confirm that lack of repression in some of these SMRT/N-CoR deletion mutants is not due to lack of appropriate protein expression, we analyzed the expression of these constructs by both *in vitro* translation and Western blot analysis after transient transfection. We find that all constructs used in this experiment express approximately equal amounts of Gal4 DBD fusion proteins *in vitro* (Fig. 4C) and that the repression-defective mutants express well *in vivo* (Fig. 4D). These results indicate that lack of repression by certain SMRT/N-CoR deletion mutants are not due to lack of protein expression.

Multiple Mechanisms of Transcriptional Repression by SMRT/N-CoR

The mechanism of transcriptional activation by nuclear receptors has been shown to require recruitment of

coactivators, including histone acetyltransferases such as CBP/p300 (36–39). The opposite of histone acetylation, histone deacetylation, has recently been implicated in transcriptional repression by unliganded receptors and the associated corepressors. Several reports have described a corepressor complex containing a Mad-dependent corepressor mSin3A, a histone deacetylase HDAC1 or mRPD3, and the nuclear receptor corepressor SMRT/N-CoR (40–48). These results suggest that histone deacetylation may be a mechanism of transcriptional repression by unliganded receptors.

To confirm the interaction between mSin3A and the defined repression domains of SMRT and N-CoR, we tested the interactions between mSin3A and the individual repression domains of SMRT/N-CoR in a mammalian two-hybrid system. Coexpression of a VP16 AD-mSin3A fusion with all Gal4 DBD-SMRT/N-CoR repression domain fusions results in weak reduction of the repression activities (Fig. 5A). Coexpression of VP16 AD-mSin3A with a Gal4 DBD-HDAC1 fusion also results in partial release of repression mediated by Gal4 DBD-HDAC1 fusion. However, no activation above the background level was observed even though a VP16 activation domain was present. Since the weak interaction between SMRT/N-CoR repression domain with mSin3A in the two-hybrid system may reflect a dominant effect of repression over activation, we tested the interaction between mSin3A and individual SMRT/N-CoR repression domains *in vitro* by far-Western analysis. Full-length mSin3A was translated and labeled *in vitro* and used as a probe for GST fusions of various SRD and NRD domains. We find that mSin3A interacts specifically and consistently with NRD-1 and NRD-4 in this assay (Fig. 5B). In one experiment, we also detected interaction between SRD-2 and mSin3A (data not shown). No interaction is observed between SRD-1, NRD-2, and NRD-3. Therefore, these results suggest that different SMRT and N-CoR repression domains may repress transcription in a mSin3A-dependent or -independent manner.

SMRT Represses Basal Transcription from Natural Promoters

The hypothesis that SMRT/N-CoR proteins are transcriptional corepressors that facilitate repression by unliganded receptors is supported by protein-protein

using 0.1 μ g (open bars), 0.2 μ g (hatched bars), or 0.5 μ g (closed bars) of plasmid DNAs. The starting and ending amino acids in each deletion construct are shown beneath each domain. SRDs, SMRT repression domains. B, Deletion mapping of the N-CoR repression domains (NRDs). The N-CoR domains are aligned with those of SMRT in panel A. The relative levels of repression were determined using 0.5 μ g plasmid DNA and comparing the result to the Gal4 DBD alone. Two new transcriptional repression domains in N-CoR were found in addition to NRD-1 and NRD-2, which were identified previously (25). C, SDS-PAGE analysis of *in vitro* translated products of SMRT/N-CoR deletion constructs used in panels A and B. Two microliters of the *in vitro* translated products were analyzed in a 12.5% acrylamide gel, which was exposed overnight. Note that most of these constructs appear to produce doublet bands, perhaps due to secondary structure of the DNA used in the translation reaction. D, Western blot analysis of the repression-defective mutants of SMRT after transient transfection into 293 cells by using anti-Gal4 DBD monoclonal antibody (0.02 μ g/ml) and detected by ECL kit. The gel on the left was resolved in a 12.5% acrylamide gel while the gel on the right was resolved in a 10% gel.

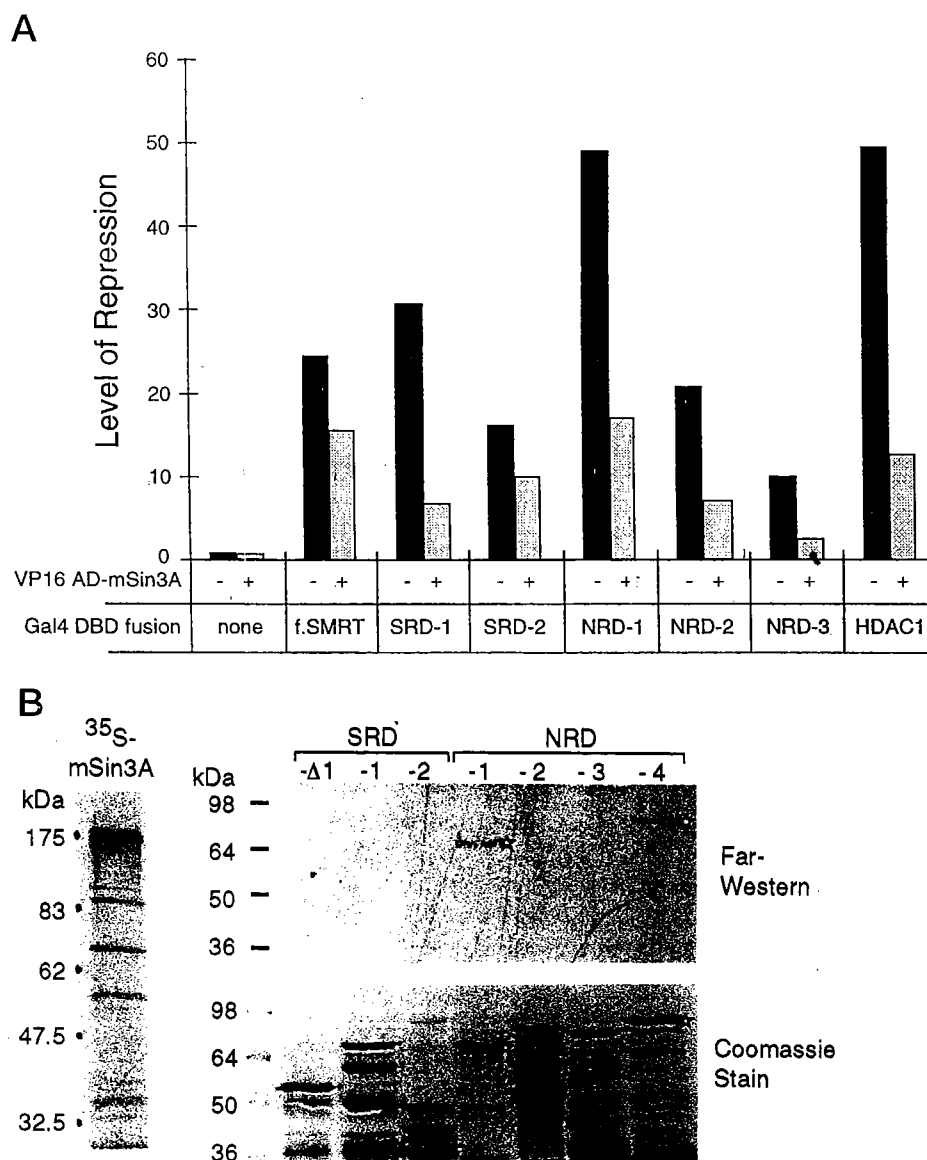


Fig. 5. Multiple Mechanisms of Transcriptional Repression by SMRT and N-CoR

A. Two-hybrid interactions of mSin3A with SMRT and N-CoR repression domains and HDAC1. The indicated Gal4 DBD fusion of SMRT and N-CoR repression domains and HDAC1 were transiently transfected into CV-1 cells together with either Gal4 AD alone or Gal4 AD-mSin3A fusion as indicated. The relative levels of repression are expressed as the means of three independent experiments relative to the Gal4 DBD alone. **B.** *In vitro* protein-protein interactions between mSin3A and SMRT/N-CoR repression domains. The GST fusions of various SRD and NRD domains were expressed in *Escherichia coli* and partially purified. The GST fusion proteins were analyzed by SDS-PAGE (right bottom panel) and examined for their abilities to interact with ³⁵S-labeled mSin3A in a far-Western blot (left upper panel). mSin3A appears to interact preferentially with intact GST-NRD-1 and GST-NRD-4 domains.

interactions and transient transfections using the Gal4 fusion system. To provide further evidence that SMRT may be physiologically relevant in transcriptional regulation, we tested the effect of SMRT overexpression on transcriptional activity of receptor-responsive promoters. Overexpression of full-length SMRT (Fig. 6, lane 2), but not that of C-SMRT lacking the repression domains (lane 3), repressed basal expression from a mouse RARβ2 promoter approximately 2-fold in com-

parison to the empty vector (lane 1). The same result is evident with two minimal response elements in the context of a thymidine kinase promoter in the absence of hormone (Fig. 5A). As expected, hormone treatment enhanced transcription from these promoters, while overexpression of full-length SMRT reduced slightly this ligand-dependent activation. C-SMRT enhances the ligand-dependent activation from these promoters (Fig. 5B). These results suggest that SMRT may, at

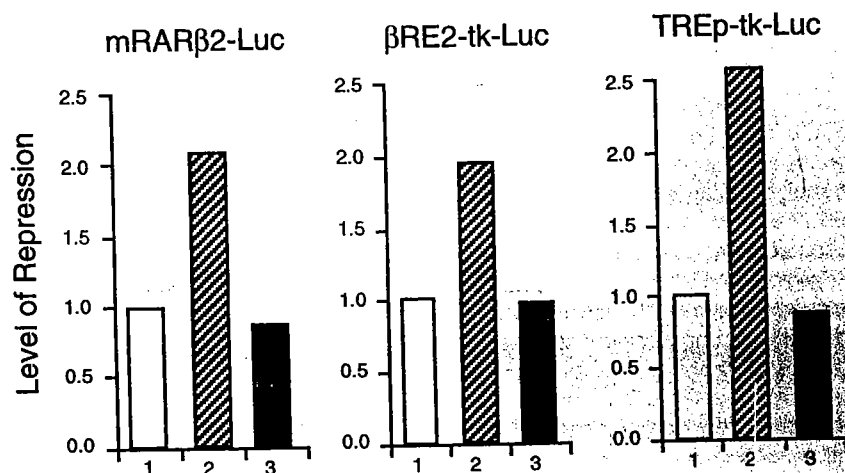
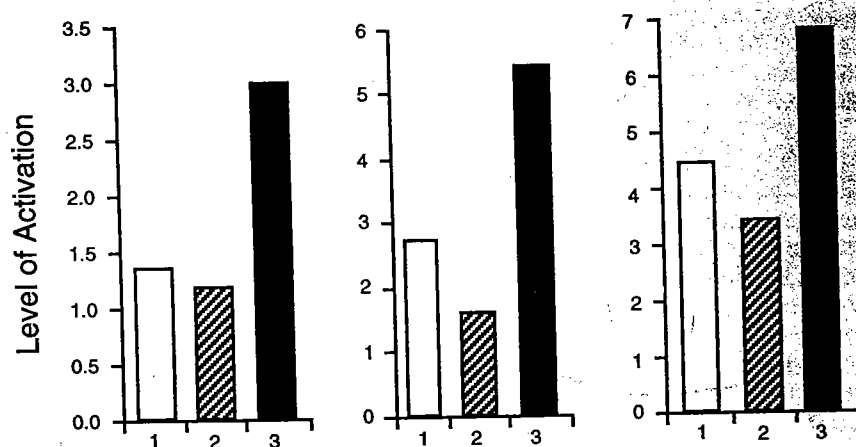
A. No hormone**B. 1μM atRA**

Fig. 6. SMRT Represses Basal Transcription from RAR- and TR-Responsive Promoters

The mRARβ2 promoter, two copies of the βRARE (βRARE-tk-luc), and the TRE (TRE-tk-luc) response elements were linked to a luciferase reporter and transiently transfected into CV-1 cells together with empty vector alone (lanes 1), full-length SMRT expression vector (lanes 2), or C-SMRT expression vector (lanes 3). The relative level of repression in the absence of hormone is shown in panel A, while the relative level of activation in the presence of 1 μM all-trans retinoic acid (atRA) is shown in panel B.

least under certain circumstances, facilitate transcriptional repression of natural promoters.

DISCUSSION

Transcriptional repression has been recognized as a critical component of TR and RAR function and is thought to be mediated by association of unliganded receptors with silencing mediators (corepressors) such as SMRT and N-CoR. To understand the function

of these putative corepressors, we have characterized their respective receptor interaction and transcriptional repression properties. Two distinct receptor-interacting domains of SMRT are identified that may interact directly with two corresponding regions in the receptor. We find that SMRT utilizes at least two distinct domains (SRD-1 and SRD-2) for transcriptional repression, consistent with a recent report (42). The two SRD-corresponding regions in N-CoR also repress basal transcription, indicating that N-CoR contains four independent repression domains. These results demonstrate the existence of multiple and

possibly redundant receptor interaction and transcriptional repression domains in SMRT and N-CoR. One might expect that this multiplicity will ensure a reliable targeting of the corepressors and appropriate repression of target genes before activation.

The hinge region of TR was originally shown to interact directly with the RID-2 region of N-CoR (25). Our results indicate that TR requires an additional C-terminal region for efficient association with SMRT. Nested deletion analyses suggest that helix 11 of the TR LBD plays an important role in stabilizing SMRT association, presumably by cooperating with the N-terminal helix 1-2 region. The interaction of SMRT with either the N terminus or C terminus of the LBD alone is very weak but detectable, suggesting that these two potential interacting surfaces may act synergistically to promote protein-protein interactions and to ensure appropriate recruitment of the corepressors. Similarly, two independent regions in the receptor have been shown to act synergistically for interaction with N-CoR (32, 49, 50). It has recently been shown that a receptor dimer is required for interaction with SMRT/N-CoR and that SMRT/N-CoR may contribute to receptor-specific transcriptional repression (51). Furthermore, an antagonist of the transcriptional activation by RXR homodimer was shown to promote association with the corepressor SMRT (52). Together, these studies suggest that SMRT and N-CoR may utilize similar but distinct mechanisms for interaction with nuclear receptors.

We cannot exclude the possibility that the tight association with SMRT by the AF2-AD deletion mutants may weaken hormone binding to the receptor, but the ability of RAR403 to respond to ligand treatment in yeast cells indicates that this mutation does not eliminate the receptor's hormone-binding capability, consistent with previous observations (14, 53). Therefore, the inability of hormone to dissociate corepressors is likely due to the lack of certain conformational changes that would normally take place in the presence of the AF2-AD. It is possible that the assumed shift of AF2-AD upon hormone binding is a prerequisite for additional structural changes that are important for corepressor dissociation. Alternatively, the shift of helix 12 may mask or compete with certain interacting surfaces required for binding corepressors. The fact that the AF2-AD deletion creates a mutant that binds tighter to the corepressors favors this model. We suspect that helix 11 could constitute such an interacting surface, since disruption of this helix eliminates the enhanced interaction resulting from deletion of AF2-AD. Our results suggest that AF2-AD may act to balance the association between nuclear receptors and the corepressors, by preventing over-association of unliganded receptors with corepressors, thereby facilitating ligand-dependent dissociation of corepressors.

Nested deletion analysis reveals two distinct subdomains in SMRT that are capable of independent interaction with nuclear receptors. These two recep-

tor-interacting domains, RID-1 and RID-2, interact differently with TR and RAR. The N-terminal RID-1 region interacts more strongly with RAR, and it contains a glutamine-rich domain, while the C-terminal RID-2 region interacts better with TR and contains a putative helical domain analogous to that identified previously in N-CoR (25). The different receptor-interacting properties of these two domains suggest that SMRT may utilize distinct mechanisms for interaction with different receptors. The RID-2 region in N-CoR has been shown to interact directly with the hinge region of TR (25), and therefore it is reasonable to predict that the N-terminal RID-1 region might interact with the C-terminal region of the LBD.

Functional analysis of the transcriptional repression activities of SMRT and N-CoR reveals two independent domains that are capable of repressing basal transcription. Together, there appear to be four independent repression domains in N-CoR and two in SMRT. These repression domains could act independently, and some repress basal transcription as efficiently as the full-length protein, suggesting that these domains might act redundantly and possibly through different mechanisms. Sequence comparison of these repression domains gives little clue as to possible mechanisms of repression. However, within SRD-1 and the corresponding NRD-3, four potential repeated motifs sharing a consensus sequence of GSITQGTGA have been identified (32). In addition, two other potential repeats with a consensus sequence of KGHVLYEG are noted. These motifs are well conserved between SMRT and N-CoR, suggesting that they might contribute to repression.

Recently, several papers reported that mSin3A and the histone deacetylase HDAC1 form a ternary complex with SMRT and N-CoR (42, 46). These results indicate that SMRT and N-CoR, while interacting with unliganded receptors, can also interact with additional corepressors such as mSin3A and mSin3B (54), as well as the histone deacetylases HDAC1 (55) and mRPD3 (56). The recruitment of histone deacetylase to target promoters by unliganded receptors through SMRT, N-CoR, and mSin3 suggests that deacetylation of histones or other factors may play a role in transcriptional repression, perhaps by establishing an unfavorable chromatin structure for transcriptional activation (41). Our results suggest weak two-hybrid interactions between SMRT/N-CoR and mSin3A, or between mSin3A and HDAC1, even though a VP16 activation domain was present. Alternatively, these results may suggest that the repression activity of the corepressor complex is dominant over that of the VP16 activation domain. An *in vitro* protein-protein interaction assay detects association of mSin3A with NRD-1 and NRD-4, but not with other repression domains. Although our results are consistent with recent reports, our data also suggest the possibility of other repression mechanisms.

MATERIALS AND METHODS

Plasmids

The GST fusions of C-SMRT (GST-SMRT) and RXR (GST-RXR) were described previously (24, 30). Serial C-terminal and N-terminal deletion mutants of human TR β and human RAR α were generated by appropriate restriction enzyme digestion and/or PCR amplification from the parental expression construct pCMX-hTR β and pCMX-hRAR α (57). The GST-SMRT deletion constructs were generated by enzyme digestion at indicated residues from the parental construct GST-SMRT. The Gal4 DBD fusions of individual repression domains of SMRT and N-CoR were generated by PCR amplification and were subsequently transferred to pGEX vector for expression of GST fusion proteins. The VP16 AD-mSin3A construct was created by subcloning the Scal (at residue 56) to Bgl/II fragment of mSin3A (58) into the pCMX-VP16 vector. Detailed information regarding these plasmids is available upon request.

Far-Western Analysis

GST fusion proteins were separated by denaturing protein gels (SDS-PAGE) and electroblotted onto nitrocellulose filters in transfer buffer (25 mM Tris-HCl, pH 8.3; 192 mM glycine; 0.01% SDS). After denaturation in 6 M guanidine hydrochloride (GnHCl), the proteins were renatured by stepwise dilution of GnHCl to 0.187 M in HB buffer (25 mM HEPES, pH 7.7; 25 mM NaCl; 5 mM MgCl₂; 1 mM dithiothreitol). The filters were then saturated in blocking buffer (5% nonfat milk, then 1% milk in HB buffer plus 0.05% NP40) at 4°C overnight or at 37°C for 1 h. *In vitro* translated ³⁵S-labeled proteins were diluted into hybridization buffer (20 mM HEPES, pH 7.7; 75 mM KCl; 0.1 mM EDTA; 2.5 mM MgCl₂; 0.05% NP40; 1% milk; 1 mM dithiothreitol), and the filters were allowed to hybridize overnight at 4°C. After three washes (5 min each) with the hybridization buffer, the bound proteins were detected by autoradiography.

Yeast Two-Hybrid Assay

The yeast two-hybrid assay was carried out in the Y190 yeast strain (59). The Gal4 DBD fusion constructs were generated in either the pAS or pGBT vector (CLONTECH, Palo Alto, CA), and the Gal4 AD fusion constructs were in the pGAD or pACT vector (CLONTECH). The β -galactosidase activities were determined with the O-nitrophenyl β -D-galactopyranoside (Sigma, St. Louis, MO) liquid assay as previously described (30).

Cell Culture and Transient Transfection

African green monkey kidney CV-1 cells were grown in DMEM supplemented with 10% resin-charcoal stripped FBS, 50 U/ml penicillin G, and 50 μ g/ml streptomycin sulfate at 37°C in 5% CO₂. One day before transfection, cells were plated in a 24-well culture dish at a density of 50,000 cells per well. Transfection was performed by standard calcium phosphate precipitation (57). All transfection experiments were performed in triplicate and were replicated at least once. Twelve hours after transfection, cells were washed with PBS and refed fresh medium containing indicated amounts of ligands. After 30 h, cells were harvested for β -galactosidase and luciferase assay as described previously (30). The relative luciferase activities are arbitrary light units normalized to the β -galactosidase activities.

In Vitro Translation and Western Blot

In vitro transcription/translation reactions were carried out in rabbit reticulocyte lysates using the TNT T7 Quick coupled

transcription/translation system (Promega, Madison, WI). [³⁵S]Methionine (Amersham, Arlington Heights, IL) was added during the translation reactions, which were performed at 30°C for 90 min. The translated reactions were analyzed by SDS-PAGE, followed by autoradiography. For Western blot analysis, transfected cells were lysed in SDS-sample buffer, and the extracts were separated by SDS-PAGE. The gels were transferred onto nitrocellulose membranes, blocked with nonfat milk, and hybridized with anti-Gal4 DBD monoclonal antibody according to manufacturer's recommendation (Santa Cruz Biotechnology, Santa Cruz, CA). The filters were washed and incubated with horseradish peroxidase-conjugated anti-mouse IgG secondary antibody and developed by enhanced chemiluminescent reaction (Amersham).

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